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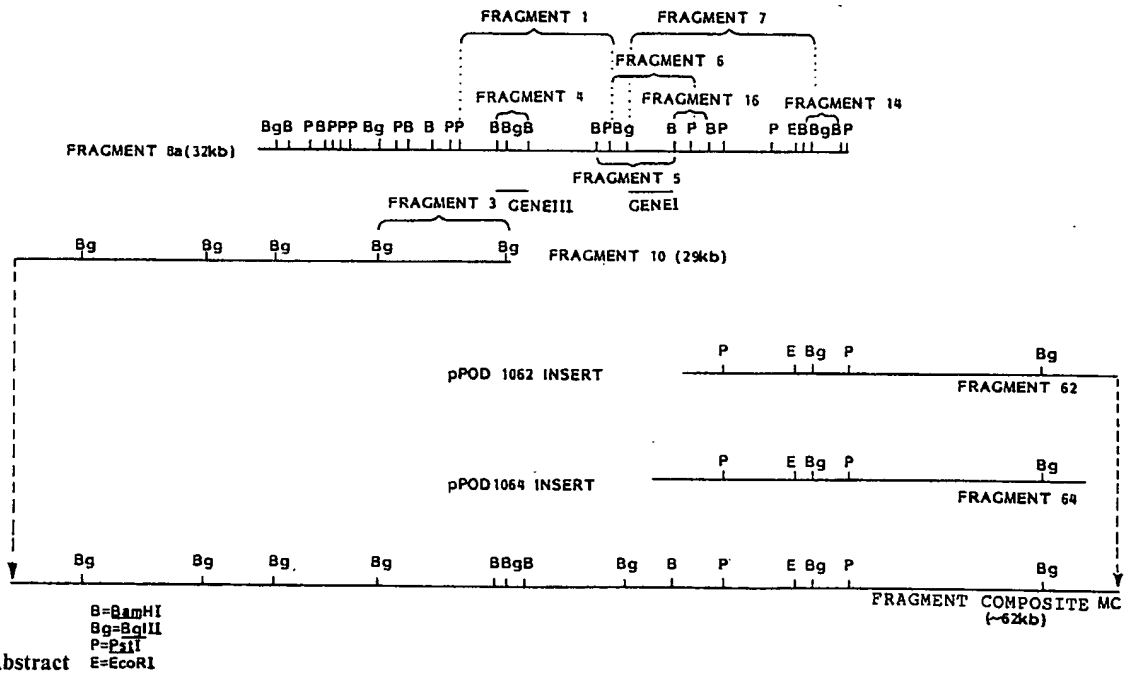

(57) Abstract

Novel methods for the isolation and characterization of genes and gene clusters involved in the biosynthesis of polyketide antibiotics. Novel DNA fragments containing such genes as well as vectors containing such genes and bacterial strains containing such vectors are prepared using these methods. The genes and gene clusters isolated by these methods are also employed in methods for the production of a polyketide antibiotic in *Streptomyces* strains that do not naturally produce the polyketide antibiotic.

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ISOLATION OF GENES FOR BIOSYNTHESIS OF POLYKETIDE ANTIBIOTICS

Field of the Invention

The present invention relates to the identification and manipulation of genes coding for enzymes involved in the biosynthesis of polyketide antibiotics (as defined below), to the use of such identified or manipulated genes in the production of polyketide antibiotics and to microorganisms containing such manipulated genes.

Background of the Invention

Polyketides are a structurally and functionaly diverse group of natural products which are related by similarities in their biosynthesis rather than by particularf structural similarities. Polyketide natural products have stgructures that are in a broad sense derived from a similar basic structure which consists of poly-β-ketomethylene chains (-$[CHRO]_n$-, where n can range from about 4 to 20). Polyketides are distinguished from other natural products by the mechanism by which this basic structure is synthesized. A schematic diagram for the polyketide pathway is presented in Figure 1. The poly-β-ketomethylene chain is synthesized from a "starter" unit, an acyl-CoA ester, by stepwise condensations of malonyl-CoA units with concomitant decarboxylation steps. The acyl-Coa ester "starter" unit varies depending on

the particular polyketide. The added malonyl units also vary with the polyketide and can be methyl malonyl, ethyl malonyl, and other alkyl malonyl groups.

It is believed that in polyketide biosynthesis the acyl and malonyl units are assembled by a multi-enzyme complex, generically called a polyketide synthase which accepts the precursor units, effects condensation and other ancillary reactions with intermediates remaining enzyme bound until the ultimate stabilized polyketide is formed and released. Only a very few polyketide synthases have been characterized; however, polyketide synthases are believed to be similar in that they incorporate a site for binding of the acyl-CoA "starter" molecule, and have transacylase functions which effect condensations. Polyketide synthases, while related, are specific to the particular polyketide that is synthesized. Polyketide synthases can display considerable differenceds in their specificity for different acyl-CoA "starter" molecules, specificity for different malonyl-CoA units, specificity for the particular order in which malonyl units are added to the chain, and for the presence of ancillary reactions occurring in association with the construction of the basic polyketide chain, including, among others, reductions, substitutions and cyclizations.

Such differences likely reflect in functional as well as structural differences in the enzymes (and in the genes encoding them) involved in the construction of the basic polyketide structure of individual polyketides. Thus, while there are some mechanistic features of polyketide biosynthesis that are common, there is no evidence that the particular enzymes involved are structurally related.

Several families of antibiotics produced by members of the actinomyces, particularly bacteria of the genus Streptomyces, belong to the polyketide group of natural products. Polyketide antibiotics include the tetracyclines (e.g., oxytetracycline), anthracyclines (e.g., daunorubicin), macrolides (e.g., erythromycin), polyenes (e.g., amphotericin B), polyethers (e.g., monensin), ansamycins (e.g., rifamycin), isochromanequinones (e.g., actinorhodin), avermectins and milbemycins ("Economic Microbiology", Vol. 3, "Secondary Products of Metabolism", Ed. Rose, A.H., 1979, Academic Press, pages 355-382; Cane, D.E. and Liang, T-C., (1983) J. Am. Chem. Soc. 105:4110-4112; and Hopwood, D.A., Malpartida, F., Keiser, H.M., Ikeda, H., Duncan, J., Fujii, I., Rudd, B.A.M., Floss, H.G. and Omura, S. (1985) Nature 314:642-644.) As seen in Figure 2, which provides structures of several polyketide antibiotics from different families, polyketide antibiotics are structurally diverse. The polyketide antibiotics represent a functionally diverse group including bacteriocides, fungicides, nematocides, insecticides and cytotoxic agents. It is believed that these antibiotics are synthesized via a polyketide pathway (vide supra, Figure 1).

Although common or structurally related precursors are used in the biosynthesis of polyketide antibiotics, it has not been shown in detail by the identification of enzymes or common intermediates that their biosyntheses follow a common pathway.

The synthesis of such polyketide antibiotics in vivo comprises a number of steps, each of which is mediated by an enzyme. Genes encoding the enzymes involved in the biosynthesis of a particular antibiotic are termed biosynthetic genes for that antibiotic. Also included among biosynthetic genes are genes which do not encode enzymes which mediate a particular

biosynthetic step but which are involved in some way in the regulation of expression of other biosynthetic genes. These regulatory genes are necessary in addition to those genes encoding biosynthetic enzymes to synthesize the antibiotic in vivo.

The generally accepted method for ascertaining the order of enzymatic steps in a biosynthetic pathway is known as "cosynthesis." This method as applied to antibiotic synthesis involves the isolation of a number of non-antibiotic-producing mutants of a microorganism which normally produces the antibiotic in question.

The order of steps is determined by ordering of mutants blocked at different steps within the pathway. The order of the blocked mutants is established in cross complementation tests in which mutants with blocks later in the pathway supply mutants with earlier blocks with precursors in the biosynthesis and thus complement antibiotic produciton in the earlier blocked mutants.

Once the sequence of steps has been established, it is possible to use genetic mapping techniques to locate the genes involved in the biosynthetic pathway. It is then possible to isolate the genes by recombinant DNA techniques.

However, these methods of investigating biosynthetic pathways are very time consuming and may not lead to an unambiguous elucidation of the pathway or to the exact location of the genes.

An example of the elucidation of a biosynthetic pathway for a polyketide antibiotic is given by Rudd and Hopwood (Rudd, B.A.M. and Hopwood, D.A., (1979) J. Gen. Microbiol. 114:35-43) who have elucidated the genetics of actinorhodin biosynthesis in Streptomyces coelicolor A3(2).

This reference also shows that the genes for actinorhodin synthesis are clustered on the bacterial chromosomal DNA. It has also been shown that the genes for the biosynthesis of other antibiotics are clustered, for instance by Ghisalba et al., in Biochemistry of Industrial Antibiotics, Ed. Van Damme, E.J. Dekker, N.Y. 1984 (rifamycin in Norcardia); Rhodes et al. (1984), J. Gen. Microbiol. 124:329, (oxytetracycline in S. rimosus); Rudd and Hopwood (1980) J. Gen. Microbiol. 119:333 (undecylprodigiosin in S. coelicolor); and Studdard et al. verbal communication to the 6th International Symposium on the Biology of Actinomycetes 1985 (chloramphenicol in S. venuzuelae).

Some methods have been proposed for isolating the genes involved antibiotic biosynthesis. One method involves a technique known as "complementation", which involves taking fragments of wild type DNA and attempting to use these fragments to restore the synthesis of the polyketide antibiotic in a blocked mutant. This method requires the isolation of antibiotic non-producing mutants.

This approach is exemplified by Malpartida and Hopwood (Malpartida and Hopwood, D.A., (1984) Nature 309:462-464), who report the isolation of a large (33 kb) contiguous segment of S. coelicolor DNA which carries the complete genetic information required for the synthesis of the antibiotic actinorhodin from simple primary metabolites, such a segment of DNA is termed an antibiotic biosynthetic gene cluster.

Another method for isolating genes involved in antibiotic biosynthesis involves cloning from a microorganism which produces a polyketide antibiotic the gene which endows the microorganism with resistance to the polyketide antibiotic. Such a resistance gene must be present when the polyketide antibiotic would otherwise be lethal to the producing microorganism. This

approach is based on the knowledge that in several cases it has been shown that the antibiotic resistance gene maps closely (is linked) with the biosynthetic genes on the chromosome. Therefore, by cloning the region around the resistance gene, it is possible to clone the clustered antibiotic biosynthetic genes. This approach is exemplified in the cloning of the erythromycin biosynthetic cluster from S. erythreus by Stanzak et al. (1985) Biotechnology 4:229-232.

Once the genes encoding biosynthesis of the antibiotic (biosynthetic cluster) have been identified and cloned, it is possible to introduce these genes into an appropriate strain of Streptomyces which does not naturally produce the antibiotic and obtain expression of the biosynthetic genes and production of the antibiotic in the normally non-producing bacterium. This has been demonstrated by Malpartida and Hopwood, 1984, who have obtained production of actinorhodin in a strain of Streptomyces parvulus which does not normally produce this antibiotic. In addition, it has been demonstrated that it is possible to produce hybrid antibiotics of medermycin, granatacin and actinorhodin, by transforming the producing various Streptomyces strains with all or some of the genes involved in the biosynthesis of actinorhodin.

Summary of the Invention

It is an object of the present invention to provide a novel method for the isolation of genes involved in the biosynthesis of polyketide antibiotics. This method is applicable to the isolation of an individual biosynthetic gene or to the isolation of polyketide antibiotic biosynthetic gene clusters. The method is particularly suited to the isolation of genes involved in the early steps of the biosynthesis of a polyketide antibiotic.

It is another object of this invention to provide a method for using the biosynthetic genes and gene clusters that are isolated by the methods described herein in order to produce a polyketide antibiotic in a strain of a bacterium of the genus Streptomyces which does not naturally produce the polyketide antibiotic. This method involves the introduction of DNA fragments containing the isolated genes or gene clusters into a suitable non-producing Streptomyces host strain wherein the introduced biosynthetic genes are expressed and the desired polyketide antibiotic is produced.

It is also an object of this invention to provide DNA fragments containing isolated polyketide antibiotic biosynthetic genes and gene clusters, DNA vectors containing these fragments and genes as well as bacterial strains which contain these vectors and DNA fragments.

The present invention therefore provides, in a first aspect, a method for isolating a gene involved in the biosynthesis of a first polyketide antibiotic which comprises the steps of: a) preparing a clone library wherein each clone contains a fragment of DNA from a microorganism which produces said first polyketide antibiotic; b) screening said clone library for hybridization to a nucleic acid probe molecule which comprises the nucleic acid sequence of at least a part of a gene involved in the biosynthesis of a second polyketide antibiotic; and c) selecting those clones which hybridize to said nucleic acid probe molecule thereby isolating a clone which contains a fragment of DNA which comprises said gene involved in the biosynthesis of said first polyketide antibiotic.

The fragment of DNA contained in the clones which are found to hybridize to the antibiotic biosynthetic gene hybridization probe can in turn be used to further probe the clone library, or a second clone library. Fragments of DNA

from hybridizing clones isolated in this manner, or combinations of such isolated fragments can then be tested for the ability to direct synthesis of the desired polyketide antibiotic in a strain of Streptomyces which does not normally produce the antibiotic. By following such a procedure it is possible to isolate and clone all the genes necessary in order to synthesize the polyketide antibiotic in a non-producing strain.

Fragments of isolated DNA which contain DNA from the microorganism that produces the first polyketide antibiotic can also be used for carrying out the method of the present invention with respect to other polyketide antibiotics. These fragments are particularly useful as probes for isolating the biosynthetic genes and gene clusters for polyketide antibiotics which are structurally similar to the first polyketide antibiotic.

The clone library or clone bank comprises preferably a library of bacteriophage, plasmid or, most preferably, cosmid clones. The source of DNA for the clone library may comprise chromosomal, plasmid or prophage DNA from a microorganism that is a producer strain of the first polyketide antibiotic.

The microorganism from which the clone library is constructed is one that carries the biosynthetic genes for the production of the first polyketide antibiotic. The microorganism can be a fungus or a bacterium, and is preferably an Actinomycete, most preferably a bacterium of the genus Streptomyces.

The hybridization probes used in this method comprise nucleic acid sequences and preferably comprise DNA.

In carrying out the isolation method of the present invention, especially where cosmid libraries are screened, a number of relatively large chromosomal

fragments may be identified. These large fragments may in some cases contain not only genes involved in the polyketide antibiotic synthesis, but genes and other DNA sequence not essential for antibiotic synthesis. It may be desirable, in such cases, to delete from such large cloned fragments, regions that are not essential for antibiotic synthesis. In the isolation of biosynthetic gene clusters it may be the case that the genes required for synthesis of the polyketide antibiotic are found on two or more DNA fragments which span a contiguous region of the chromosome. In this case, it may be desirable to ligate the contiguous fragments together in order to construct a single DNA fragment which contains all of the required biosynthetic genes. It may also be desirable to delete non-essential regions from such a composite DNA fragment.

In one specific embodiment, the isolation method of the present invention employs hybridization probes which comprise the nucleic acid sequence of at least part of a gene involved in an early step of the biosynthesis of the second polyketide antibiotic.

In another specific embodiment, the isolation method of the present invention employs hybridization probes which comprise the nucleic acid sequence of at least a part of a gene involved in the biosynthesis of actinorhodin, particularly probes comprising the nucleic acid sequence of at least part of actinorhodin Gene I or actinorhodin Gene III.

In another specific embodiment, the isolation method of the present invention employs hybridization probes which comprise the nucleic acid sequence of at least a part of a gene involved in the biosynthesis of milbemycin, particularly probes comprising the nucleic acid sequence of at least part of milbemycin Gene I or milbemycin Gene III.

The present invention also provides, in a second aspect, a method for producing a first polyketide antibiotic in a naturally non-producing strain of a bacterium of the genus Streptomyces which comprises the steps of:

a. isolating a DNA fragment which comprises the clustered biosynthetic genes for said first polyketide antibiotic from a strain of a bacterium of the genus Streptomyces which produces said first polyketide antibiotic wherein said isolation comprises the steps of:

i. preparing a clone library wherein each clone contains a fragment of DNA from a microorganism which produces said first polyketide antibiotic;

ii. screening said clone library for hybridization to a nucleic acid probe molecule which comprises the nucleic acid sequence of at least a part of a gene involved in the biosynthesis of a second polyketide antibiotic;

iii. selecting those clones which hybridize to said nucleic acid probe molecule thereby isolating a clone which comprises a first DNA fragment which comprises at least part of the biosynthetic gene cluster of said first polyketide antibiotic;

iv. testing said first selected DNA fragment which comprises at least part of the biosynthetic gene cluster of said first polyketide antibiotic for the ability to direct synthesis of said first polyketide antibiotic in said non-producing strain of Streptomyces;

v. rescreening said clone library or a second clone library with a nucleic acid probe which comprises the right or left terminal region of said first selected DNA fragment;

vi. selecting those clones which hybridized to the right or left terminal region of said first selected DNA fragment thereby isolating a clone which comprises a second DNA fragment which comprises chromosomal DNA sequences to the right or left of said first selected clone;

vii. repeating said testing, rescreening and selection steps employing said second selected DNA fragment and subsequent selected DNA fragments until said DNA fragment which comprises the clustered biosynthetic genes for said first polyketide antibiotic is isolated as ascertained by production of said first polyketide antibiotic in said nonproducing strain of Streptomyces in said testing step; and

b. introducing said cloned fragment containing said cluster of biosynthetic genes into said non-producing strain of a bacterium of the Streptomyces, thereby producing said polyketide antibiotic in said non-producing strain of Streptomyces.

This method employs DNA fragments containing biosynthetic genes and gene clusters which are obtained by the isolation methods of the present invention. By applying the iterative technique of the present invention DNA

fragments containing the entire biosynthetic gene cluster for a polyketide antibiotic can be isolated and introduced into a non-producing strain in order to effect synthesis of the polyketide antibiotic.

Bacterial hosts which are suitable for use in this method are strains of bacteria of the genus Streptomyces which do not naturally produce the desired polyketide antibiotic. Suitable host strains are those that synthesize the necessary biochemical precursors for synthesis of the polyketide antibiotic, e.g. the appropriate acyl-CoA and malonyl-CoA precursors, and that are transformable. It is contemplated that strains of Streptomyces lividans, Streptomyces ambofaciens, Streptomyces coelicolor, Streptomyces avermitilis and Streptomyces parvulus will be particularly useful in this method. It may be that different Streptomyces host strains will be more or less preferably employed in this method dependent on the particular polyketide antibiotic to be synthesized.

One specific embodiment of the polyketide antibiotic production method of the present invention involves the production of milbemycins, particularly by introduction of the milbemycin biosynthetic gene cluster, or more particularly by introduction of DNA fragment MC or a DNA fragment that is functionally equivalent to fragment MC into a milbemycin non-producing strain of Streptomyces. It is contemplated in this embodiment that Streptomyces lividans is a suitable host strain.

Another specific embodiment of the present antibiotic production method involves production of avermectins in an appropriate host bacterium.

The present invention provides, in another aspect, a method for activating the expression of a polyketide antibiotic biosynthetic gene cluster

in a bacterial strain of the genus Streptomyces which strain contains the biosynthetic genes sufficient for production of the polyketide antibiotic but lacks a functional polyketide antibiotic biosynthesis activator gene which comprises introducing into said bacterial strain a DNA fragment which comprises milbemycin Gene II.

In particular it is contemplated, in a specific embodiment, that this method for activating expression of synthesis of a polyketide antibiotic can be applied to synthesis of actinorhodin, milbemycin and avermectin and particularly to the biosynthesis of one of these antibiotics in strains of Streptomyces lividans, Streptomyces coelicolor and Streptomyces avermitilis.

It is also contemplated that this method may also be employed to enhance the level of production of a polyketide antibiotic by introduction of milbemycin Gene II into a strain which naturally produces the polyketide antibiotic.

The present invention provides, in yet another aspect, DNA fragments and vectors containing these fragments which are useful in the methods of the present invention. In particular, vectors are provided which comprise DNA fragments which consist essentially of the milbemycin biosynthetic gene cluster, preferably where the DNA fragment is fragment MC. Vectors which comprise DNA molecules consisting essentially of the DNA sequence encoding a milbemycin biosynthetic gene are also provided, including those encoding milbemycin Gene I, milbemycin Gene II and milbemycin Gene III. Vectors which comprise DNA molecules that are DNA fragment 1, fragment 2, fragment 3, fragment 4, fragment 5, fragment 6, fragment 7, fragment 8a, fragment 8b, fragment 10, fragment 12, fragment 14, fragment 16, fragment 62 and fragment 64 are also provided. The vectors and DNA fragments provided herein are

useful as hybridization probes or in some cases for introduction into appropriate host bacterial in order to effect synthesis of polyketide antibiotics or to effect a modification of a polyketide antibiotic naturally produced by the host strain.

With respect to the hybridization probes provided in the present invention it is contemplated that in addition to the specific fragments identified, fragments that are functionally equivalent as hybridization probes to the specific DNA fragments identified are also part of the present invention. This includes hybridization probes that consist essential of the sequences of the fragments identified herein, as well as hybridization probes whose sequences are derived from the sequences of the fragments identified herein.

The present invention also provides a method for investigating the structure of a gene cluster for the synthesis of a first polyketide antibiotic comprising:

a) infecting a microoganism which is capable of synthesizing the polyketide antibiotic with a phage vector having a selectable marker, such as resistance to an antibiotic which is fatal to the untransformed microorganism, but having no attachment site, the page vector having inserted therein adjacent its antibiotic resistance gene a gene fragment which is suspected of being involved in the biosynthesis of the first polyketide antibiotic;

b) selecting the lysogens so formed by repeated growth on a medium containing the fatal antibiotic; and

c) screening the selected microorganisms for production of the first polyketide antibiotic.

An approach similar to this has already been described by Chater and Bruton (Chater, K.F. and Bruton, C.J. (1983) Gene 26:67-78). However, the results given by the present investigation method are surprisingly and unexpectedly different from those seen by Chater and Bruton.

The expected result, as shown by Chater and Bruton, depends on the location of the gene fragment inserted in the phage. If the fragment is within a chromosomal transcription unit, it would be expected that in transformants, in which homologous recombination has caused the phage DNA to be integrated into the chromosomal DNA, disruption of transcription would occur, and no production of the antibiotic would be observed. Conversely, if the fragment includes a complete transcription unit, but no other parts of other transcription units, no disruption of transcription would be expected.

It has surprisingly been found that, by carrying out the above investigation method, antibiotic production may be stopped whether or not the gene fragment comprises a complete transcription unit. Moreover, in some cases, whether antibiotic production is stopped is dependent on the orientation in the chromosomal DNA of the inserted phage DNA. This is not what would be expected from the work carried out by Chater and Bruton.

Investigation of this unexpected result shows that, during growth on the medium containing the fatal antibiotic, deletion of dna downstream of the antibiotic resistance gene takes place. This deletion removes not only the phage DNA and the duplicate copy of the inserted gene fragment, but also some of the chromosomal DNA.

If the gene fragment is located towards the center of the antibiotic biosynthetic gene cluster, deletion in either direction is likely to disrupt

antibiotic synthesis, and in the majority of transformants no antibiotic production will be observed. If the gene fragment is located outside the gene cluster, deletion in either direction is unlikely to affect antibiotic production.

If the gene fragment is located towards the end of the gene cluster, transformants having deletions in one direction may not affect antibiotic production, since the deletions will take place outside the gene cluster. However, transformants having deletions in the other direction may have antibiotic production disrupted by deletions occurring within the gene cluster.

Thus, the above investigation method can be used to determine whether a cloned gene fragment is actually within the desired gene cluster and to delimit the extent of the gene cluster.

The present invention, in all its aspects also enables the skilled person readily: to investigate the biosynthesis of any particular polyketide antibiotic, which may lead to an improvement of the yield of the antibiotic; to investigate the effect of using genes from one microorganism on the biosynthesis of a polyketide antibiotic in another microorganism; or to produce novel polyketide antibiotics by insertion into a microorganism of combinations of genes or parts of genes isolated from different microorganisms. Alternatively, novel polyketide antibiotics could be produced by causing mutations in isolated genes or by recombining parts of various genes, using conventional DNA techniques, and inserting the manipulated genes into a microorganism.

Brief Description of the Figures

Figure 1 is a schematic diagram of the polyketide pathway.

Figure 2 shows the structural formulae of four representative polyketide antibiotics;

Figure 3 provides restriction enzyme maps of the S. avermitilis DNA fragments inserted into plasmids pIJ 2303, pIJ 2305 and pIJ 2308; the location of certain actinorhodin genes within these inserts is also provided (Malpartida and Hopwood, 1986).

Figure 4 shows the results of Southern hybridization experiments using the pIJ 2305 fragment as a probe of PstI digests of total chromosomal DNA of (1) S. coelicolor, (2) S. lividans and (3) Streptomyces sp. B41-146.

Figure 5 shows the restriction map of vector pIJ 610;

Figure 6 shows the restriction map and relative arrangement of the fragments 8a, 8b and 10. The location of fragments 1, 2, 3, 4, 5, 6, 7, 14 and 16 is also provided.

Figure 7 shows the scheme for transferring the clone 8a insert into vector pIJ 922.

Figure 8 shows the restriction map and relative arrangement of the fragments 8a, 62 and 64.

Figure 9 shows a restriction map of the phage vector pPOD9.

Figure 10 shows the theoretical insertion of pPOD11 into the Streptomyces sp. B41-146 chromosome compared to the structure of one actual insertion event.

Figure 11 shows the theoretical insertion of pPOD12 into the Streptomyces sp. B41-146 chromosome compared to the structure of one actual insertion event.

Figure 12 shows the theoretical insertion of pPOD1071 into the Streptomyces sp. B41-146 chromosome compared to the structure of one actual insertion event.

Figure 13 shows the theoretical insertion of pPOD1072 into the Streptomyces sp. B41-146 chromosome compared to the structure of one actual insertion event.

Detailed Description of the Invention

The present invention is based on the hypotheses that structurally similar condensation enzymes are involved in at least the early steps of the biosyntheses of polyketide antibiotics and that the several genes required for the biosynthesis of an individual polyketide antibiotics are clustered in the DNA of the microorganism that produces that polyketide antibiotic.

The group of polyketide natural products are defined by an overall similarity in the biosynthetic pathway by which they are synthesized in vivo and similar precursors may be involved in the synthesis of the basic polyketide chain. There is, however, much structural diversity among polyketides in general and specifically among the various polyketide antibiotics. The structurally diversity of the polyketide antibiotics reflects differences in their biosynthetic pathways. It is believed that major structural variation of the stabilized polyketide core are mediated not only by subsequent enzymatic modification of the basic polyketide chains but

in most cases by a variety of enzymatic activities that are directly associated with the synthesis of the basic polyketide chain.

It has not been demonstrated that any of the enyzmes involved in the biosynthesis of any polyketide antibiotic are similar in function or structure to those involved in the biosynthesis of another polyketide antibiotic. It has certainly not been shown that there is any structural similarity in the protein sequence or the gene coding sequences of enzymes associated with the biosynthesis of different polyketide antibiotics.

Further it is known that the condensation and other enzymes that function in the biosynthesis of other polyketide natural products are also functionally similar to those of polyketide antibiotic synthesis, suggesting that the early steps in the biosynthesis of many polyketides, including for example those of fatty acid biosynthesis may be mediated by functionally and therefore potentially structurally similar enzymes.

An important aspect of the present work is the demonstration that DNA fragments which encode genes (or parts of genes) involved in the biosynthesis of one polyketide antibiotic hybridize to DNA which encodes genes (or parts of genes) involved in the biosynthesis of a second polyketide antibiotic. This is demonstrated herein for two polyketides that are not structurally similar and which are representatives of two different families of polyketide antibiotics. In particular, it is demonstrated that DNA fragments containing actinorhodin biosynthetic genes specifically hybridize to DNA fragments of a strain of Streptomyces, Streptomyces sp. B41-146, which produces the nematocidal polyketide antibiotic milbemycin (Figure 4). DNA hybridization is demonstrated between the actinorhodin biosynthetic genes, Gene I and Gene III, and Streptomyces sp. B41-146 DNA fragments that have been shown to contain genes involved in the biosynthesis of milbemycin.

For reason noted above, the fact that it is possible to identify DNA fragments containing biosynthetic genes for one polyketide antibiotic by hybridization to probes containing biosynthetic genes for a structurally unrelated polyketide antibiotic is surprising and unexpected. It would be expected either that no hybridizing sequences would be identified or more likely that the hybridizing sequences so identified would not be specifically associated with the biosynthesis of the desired polyketide antibiotic.

Actinorhodin Gene I and Gene III are found to be blocked at the earliest steps in the biosynthetic pathway of actinorhodin (Malpartida and Hopwood, 1986). It is believed that these genes may be associated with the actinorhodin polyketide synthase. By analogy to the actinorhodin genes, the specific Streptomyces sp. B41-146 DNA that hybridizes to act Gene I is designated milbemycin Gene I and that which hybridizes to act Gene III is designated milbemycin Gene III. Again by analogy to these actinorhodin genes, it is believed that milbemycin Gene I and milbemycin Gene III are associated with early steps in the biosynthesis of milbemycin, possibly associated with milbemycin polyketide synthase.

The actinorhodin genes were in fact employed as probes in hybridization screens of a library of Streptomyces sp. B41-146 cloned DNA. Hybridization screens using an act Gene III probe resulted in the isolation of several DNA fragments (fragments 8a, 8b and 10, Figure 6). It was demonstrated by restriction enzyme mapping that these B41-146 DNA fragments overlap one another and span an approximately 48 kb long contiguous region. Act Gene III probe hybridization within this region was localized to a 1.6 kb BamHI restriction fragment, numbered fragment 4. The act Gene III probe also hybridized to fragments 1, 2 and 3 which all include fragment 4 sequence.

Using similar techniques act Gene I probe hybridization was localized within the cloned B41-146 DNA to fragments 8a, 8b, 5, 6, and 7. Fragment 10 did not hybridize to act Gene I sequences. The regions that hybridize to both act probes are contained on fragment 8a.

Table 1 presents the results of complementation experiments, in which B41-146 fragments containing sequences that hybridized to act Gene I and act Gene III sequences were introduced into Streptomyces coelicolor mutants blocked in act Gene I (TK 17) or act Gene III (TK 18). As seen in Table 1, the B41-146 fragments contain DNA sequences which appear to functionally complement both act Gene I and Act Gene III and the complementary sequences correspond to those regions showing hybridization to the actinorhodin gene probes. The interpretation of the results of Table 1 are complicated, however, by the leaky phenotype of the TK 17 mutant.

As part of the characterization of the B41-146 fragments isolated in hybridization experiments, subfragments of the isolated DNA were introduced into Streptomyces lividans. This Streptomyces species contains the genes for the production of actinorhodin but does not normally produce this polyketide antibiotic. The presence of actinorhodin genes in S. lividans DNA has been confirmed by the presence within S. lividans populations of a small number (usually less than 1%) of individuals which produce actinorhodin. These individuals are likely to be spontaneous mutants.

It was found that when B41-146 fragment 7 was inserted in either orientation or when fragment 12 was inserted in one orientation that all transformed S. lividans produced actinorhodin. The B41-146 DNA fragments therefore contain sequences that activate synthesis of the polyketide antibiotic actinorhodin. It has also be demonstrated that fragment 7 contains

sequences, a gene, that functionally complements a Streptomyces coelicolor act Gene II mutation. The act Gene II is believed to be involved in the regulation of actinorhodin biosynthesis. The B41-146 DNA sequence that functionally complement act Gene II are designated milbemycin Gene II and are by analogy expected to be involved in the regulation of milbemycin biosynthesis. Fragment 7 does not show hybridization to act Gene II sequences, and is therefore not structurally similar to the actinorhodin regulatory gene. Act Gene II also activates actinorhodin production in transformed S. lividans. Horinouchi and Beppu (1984) Agric. Biol. Chem. 48:2131-2133 have reported the isolation of a regulatory gene, afsB, which controls biosynthesis of A-factor and which also controls the production of the red pigments, actinorhodin and prodigiosin. There is at present no indication that afsB is equivalent to either act Gene II or milbemycin Gene II.

It may be the case that many strains of Streptomyces contain silent biosynthetic genes for secondary metabolite production, including polyketide antibiotic production. The milbemycin Gene II sequence and fragments containing it are useful in the activation of such silent polyketide antibiotic biosynthetic genes in strains like S. lividans and may be widely applicable in other Streptomyces species. The milbemycin Gene II sequence may also be useful in the enhancement of the level of production of a polyketide antibiotic in a Streptomyces strain that naturally produces the antibiotic.

The presence of milbemycin Gene II sequence at the far right hand end of fragment 8a, suggests that other genes associated with the biosynthesis of milbemycin could be found in the B41-146 chromosome in the region adjacent to the right of the region spanned by fragment 8a. In order to isolate and clone

this adjacent chromosomal DNA, a subfragment (fragment 14) at the far right end of 8a was selected for use a hybridization probe for a second clone library of B41-146 DNA. Two clones were selected in such hybridization screens which contained inserts which extended in the rightward direction from the end of fragment 8a. These fragments are called 62 and 64 and are shown compared to fragment 8a and 10 in Figure 8. Similar techniques can be employed to isolate DNA sequences which extend in the leftward direction from a desired fragment. Application of such techniques has been termed chromosome "walking".

Fragments 10, 8b, 8a, 62 and 64 define and span a chromosomal region of about 62 kb in length. It is believed that this region contains the complete biosynthetic gene cluster for the production of milbemycin. A composite fragment, fragment MC can be constructed by appropriate restriction enzyme cleavages, ligations and isolation of any intermediate DNA fragments and the desired MC fragment by conventional DNA isolation methods. Fragment MC spans 62 kb of DNA; however, by analogy with other polyketide antibiotics for which the complete biosynthetic gene cluster has been cloned, the milbemycin gene cluster should range in size between about 30 to 40 kb of DNA. It is therefore likely that fragment MC contains in addition to the milbemycin biosynthetic gene cluster, other genes not involved in the biosynthesis. It is likely, based on the mapped locations of the milbemycin Gene I, II and III regions that DNA regions that are not essential for milbemycin production are located at the leftward end of fragment MC. Delineation of the region essential for milbemycin biosynthesis can be done by iterative removal of sequences at the left or right end of fragment MC coupled with intermediate testing of the resultant shorter DNA fragment for the ability to direct synthesis of milbemycin in a bacterial host which does not naturally produce

milbemycin (e.g., S. lividans). Fragment MC and fragments derived therefrom that are essentially equivalent in function to fragment MC in that they contain the complete biosynthetic gene cluster for milbemycin production are useful in methods described herein for the production of milbemycin in non-milbemycin producing strains of Streptomyces.

An attempt was made to further characterize the milbemycin biosynthetic gene cluster by use of an insertional inactivation techniques which had been previously described by Chater and Burton (1983) Gene 26:67-78. However, the results of these experiments were unexpectedly different from those described by Chater and Burton. In this method, cloned DNA fragments are inserted into a defective ΦC31 vector in which the attachment site has been deleted. Phage insertion into the chromosome of an infected strain results through homologous recombination mediated by the inserted cloned DNA. Recombination occurs at the chromosomal sequences which hybridize to the cloned DNA. Theoretically, phage insertion will only cause the disruption of gene expression when the cloned DNA is internal to a transcription unit (i.e. contains neither the 5' nor the 3' end sequences of the transcription unit). It was found, however, using the DNA fragments and strains described herein that phage insertion most often induced disruption of gene expression. This was observed even with cloned DNA fragments, like fragment 4, which were believed to contain entire transcription units. It was found that this technique induced the deletion of both phage as well as chromosomal DNA. The technique therefore proved to be method for the production of milbemycin non-producing mutants. The results of these experiments coincidentally provided another demonstration that the cloned B41-146 DNA of fragment MC contained genes associated with milbemycin production, since deletion of chromosomal DNA in this region resulted in non-producing mutants.

The techniques applied herein to the isolation of the milbemycin biosynthetic gene cluster define a general method for the isolation of polyketide antibiotic biosynthetic gene clusters. This method is an extension of the method described above for the isolation of polyketide antibiotic biosynthetic genes which comprises, after the initial selection of clones which hybridize to probes containing a gene or part of a gene involved in the biosynthesis of another polyketide antibiotic, iterative testing and rescreening steps by which chromosomal sequences adjacent to the initially selected DNA fragments are cloned. The testing and rescreening steps are continued until the entire biosynthetic gene cluster is cloned, which is demonstrated by the detection of the production of the polyketide antibiotic in a naturally non-producing Streptomyces strain into which the cloned DNA region has been inserted. It may be desirable to ascertain by the use of appropriate controls that the introduced cloned DNA is directing synthesis of the polyketide antibiotic.

Once isolated, the polyketide antibiotic biosynthetic cluster can be employed for the production of the antibiotic in a suitable strain that does not naturally produce the antibiotic. It may be desired to produce an antibiotic in a heterologous host, because the chosen host provides some benefit for the production of the antibiotic, for example faster or more efficient growth or ease of isolation of the product antibiotic from growth medium.

Fragments of B41-146 DNA that contain milbemycin Gene I and milbemycin Gene III, particularly fragments 4 and 5, are expected to be useful, as were the act Gene I and Act Gene III probes, as hybridization probes in the isolation of biosynthetic genes of a variety of polyketide antibiotics. These

fragments and also other B41-146 DNA fragments in the vicinity of the Gene I and Gene III sequences are expected to be particularly useful in the isolation of biosynthetic genes for polyketide antibiotics which are structurally similarly to milbemycin, for example, avermectins.

It has recently been reported (Malpartida et al. Nature, submitted November, 1986) that the act Gene I and act Gene III sequences show specific hybridization to DNA from a number of polyketide antibiotic producing strains of Streptomyces, including strains which produce the anthracyclines: tertacenomycin and adriamycin; the macrolides: spiramycin, tylosin, pikromycin and oleandomycin; the polyethers: nonactin, salinomycin, lasalocid and monensin; as well as other isochromanequinone antibiotics. This report further supports the contention that the act Gene I, act Gene III, milb Gene I and milb Gene III probes will be generally useful for the isolation of biosynthetic genes of a variety of polyketide antibiotics.

The hybridization methods described herein can be applied to the the isolation of biosynthetic genes for a variety of polyketide antibiotics. The methods of the present invention enable the skilled person to identify and isolate the genes for polyketide antibiotic biosynthesis without having to follow the lengthy and tedious prior art process of mutant preparation, complementation and gene mapping. Moreover this method can be applied in cases where mutant preparation is particularly onerous, as when the screening method for non-producing mutants involves chemical analysis rather that a more simple plate assay. Further, such a method can be applied in cases where there is no resistance gene associated with antibiotic biosynthesis, for example in those cases where the antibiotic is not toxic to bacteria (milbemycins and avermectins).

The methods of the invention involve the use of recombinant DNA techniques which are known per se and therefore do not form part of the invention. For instance, the preparation of clone libraries and the technique of hybridization screening are well known to a person skilled in the art. Thus, a skilled person will be able to carry out the methods of the present inventions using only his ordinary skill and knowledge of the art. A number of techniques that are standard in the art are described in: Maniatis et al. (1982) Molecular Cloning, Cold Spring Harbor Laboratory, Cold Spring Harbor, New York; Wu (ed.) (1979) Meth. Enzymol. 68: Wu et al. (eds.) (1983) Meth. Enzymol. 100 and 101; Grossman and Moldave (eds.) Meth. Enzymol. 65; Miller (ed.) (1972) Experiments in Molecular Genetics, Cold Spring Harbor Laboratory, Cold Spring Harbor, New York; Old and Primrose (1981) Principles of Gene Manipulation, University of California Press, Berkeley; Schleif and Wensink (1982) Practical Methods of Molecular Biology; Glover (ed.) (1985) DNA Cloning Vol. I and II, IRL Press, Oxford, U.K.; Hames and Higgins (eds.) (1985) Nucleic Acid Hybridization, IRL Press, Oxford, U.K.

Example 1: Hybridization using actinorhodin biosynthetic gene probes

Malpartida and Hopwood (1984) Nature 309:462-464 have reported a recombinant plasmid pIJ2303 which contains a fragment of S. coelicolor DNA approximately 33 kb in length which contains the genes involved in biosynthesis of the polyketide antibiotic actinorhodin. A restriction enzyme map of the insert of pIJ2303 is shown in Figure 3, which also includes the locations of numbered actinorhodin genes within the DNA insert (Malpartida and Hopwood (1986) Mol. Gen. Genet. 205:66-73). Two plasmids, which contain subfragments of the pIJ2303 insert, designated pIJ2305 and pIJ2308 were obtained from BglII and PstI digests, respectively, of pIJ2303. Figure 3 also

provides the restriction maps of the pIJ2305 and pIJ2308 inserts as well as the physical location of actinorhodin genes within these inserts.

PstI digests of DNA derived from S. coelicolor, S. lividans and S. hygroscopicus subsp. aureolacrimosus were probed using the Southern hybridization technique with radioactively labelled insert of pIJ2305. S. coelicolor and S. lividans contain the genes for actinorhodin biosynthesis. S. hygroscopicus subsp. aureolacrimosus, which is commonly known as Streptomyces sp. B41-146 (available as NRRL No. 5739), produces the polyketide antibiotic milbemycin (see Figure 2 for structure). Southern hybridization was performed by standard techniques (Maniatas et al. (1982) Molecular Cloning Cold Spring Harbor Laboratory; Hames and Higgins (eds) (1985) Nucleic Acid Hybridization IRL Press Oxford). More specifically, filters were prehybridized for 2 hrs at 37°C in a solution containing 6x SSC (Salt-Sodium Citrate, where 1xSSC is 0.15M NaCl, 0.15M trisodium citrate, pH 7.0)/ 50% v/v formamide; 0.1% wt/v SDS (Sodium dodecyl sulfate) and 50 µg/ml salmon sperm DNA. Hybridization was performed at 37°C, overnight using the same solution. Filters were washed twice. First at 37°C for 1 hr. with a 6xSSC/ 50% v/v formamide/0.1% wt/v SDS solution followed by a second wash at 37°C for 40 min. with 6xSSC.

Southern hybridization results are shown in Figure 4. The pIJ 2305 insert probe hybridized to S. coelicolor DNA PstI fragments of 9.1, 3.4, 2.9, 2.3 and 1.6 kb, as expected. A similar hybridization pattern was observed with S. lividans DNA fragments. The actinorhodin gene probe insert of pIJ2305 also hybridized to several fragments of Streptomyces sp. B41-146 DNA, having sizes of approximately 8, 7 and 4 kb.

In order to identify the region of insert pIJ2305 which hybridized to Streptomyces sp. B41-146 DNA fragments, BamHI fragments of the pIJ2305 insert were subcloned, labelled and used as probes in hybridization experiments with Streptomyces sp. B41-146 DNA digests. In these Southern hybridizations, the prehybridization and hybridization steps were performed as above. However, filters were washed at 37°C for 1 hr using 6xSSC/ 50% v/v formamide/ 0.1% wt/v SDS followed by two subsequent 1 hr washes at 37°C using 2x SSC/ 50% v/v formamide. These hybridization experiments showed conclusively that the 1.1 BamHI fragment of the pIJ2305 insert which contains the entire transcription unit for actinorhodin Gene III (Figure 3) hybridizes specifically to an approximately 8.0 kb fragment of Streptomyces sp. B41-146 DNA.

A second actinorhodin gene probe specific for Gene I was prepared by digesting the pIJ2308 insert with BamHI and isolation of a 2.2 kb fragment which contains actinorhodin Gene I.

Example 2: Hybridization screening of a Streptomyces sp. B41-146 clone library with actinorhodin gene probes

A cosmid library of Streptomyces sp. B41-146 DNA was prepared by Sau3a digestion to give 20-30 kb fragments. The fragments were cloned into the BglII site of the shuttle cosmid pIJ 610, which can be obtained from Tobias Kieser, John Innes Institute, using in vitro packaging techniques in Escherichia coli. A restriction map of vector pIJ 610 is shown in Figure 5. Selection for inserts is made on the basis of in vitro packaging size constraints in conjunction with phosphatased vector.

The cosmid library thus prepared was screened by hybridization to the 1.1 kb BamHI fragment containing actinorhodin Gene III (vide supra). Conventional techniques were used. Specifically, filters were prehybridized at 37°C for 2.5 hr using a solution of 6xSSC/ 50% v/v formamide/ 0.1% SDS/ 50 µg/ml salmon sperm DNA. Hybridization was carried out overnight at 37°C using the same solution. Filters were washed at 37°C for 70 min. using 6xSSC/ 50% v/v formamide. Of 1,000 clones screened, 3 were found to hybridize to the actinorhodin Gene III probe. These clones were designated 8a, 8b, and 10. The fragments of Streptomyces sp. B41-146 DNA contained in these clones were also designated 8a, 8b and 10. Restriction maps of these fragments are shown in Figure 6. The approximate sizes of fragments 8a, 8b and 10 are 32 kb, 29 kb and 29 kb respectively. Using conventional restriction enzyme analysis it was shown that these fragments overlap one another and span a contiguous region of Streptomyces sp. B41-146 DNA of about 48 kb.

Restriction subfragments of 8a, 8b and 10 were screened for hybridization to the 1.1 BamHI actinorhodin Gene III specific probe. Southern filters were prehybridized for 2 hrs as in Example 1 and hybridization was carried out overnight, again as in Example 1. Filters were washed twice at 37°C. The first wash was for 1 hr using 6x SSC/ 50% v/v formamide, the second wash was for 1 hr using 2x SSC/ 50% v/v formamide. The following fragments were found to hybridize to the Gene III specific probe:

An 8 kb PstI subfragment of both 8a and 8b, designated fragment 1, approximately 4 kb of which is also present in 10;

A 13 kb BglII subfragment of 8a and 8b, designated fragment 2;

A 7 kb BglII subfragment of 10, designated fragment 3; and

A 1.6 kb BamHI subfragment of 8a and 8b, designated fragment 4, approximately 1 kb of which is also present in 10. The location of fragments 1-4 is provided in Figure 6.

The fragments 8a, 8b and 10 and restriction subfragments thereof were also analyzed for hybridization to the 2.2 kb BamHI actinorhodin Gene I specific probe (vide supra). Again conventional Southern hybridization techniques were employed. Specifically, filters were prehybridized for 2 hr and hybridization was conducted at 37°C overnight as described above. Filters were washed at 37°C three times: first for 1 hr with 6x SSC/ 50% v/v formamide; then for 90 min with 2x SSC/ 50 % v/v formamide and then for 90 min with 1x SSC/ 50% v/v formamide. The Gene I specific probe was found to hybridize to both fragments 8a and 8b, but not to fragment 10. Within fragments 8a and 8b, the probe was found to hybridize to a 4.2 kb BamHI subfragment, designated fragment 5; a 4.2 PstI subfragment, designated fragment 6; and a 10 kb BglII subfragment, designated fragment 7 (see figure 6).

It can be seen in Figure 6 that fragment 8a includes sequences that hybridize to both actinorhodin gene specific probes.

Example 3: Complementation of S. coelicolor actinorhodin(-) mutants with Streptomyces sp. B41-146 DNA fragments

Almost the entire insert from clone 8a was subcloned as a 27 kb BglII fragment, fragment 12 in both orientations into plasmid pIJ 922, as shown in Figure 7. Plasmid pIJ 922 (Lydiate et al. (1985) Gene 35:223-235) is a low copy number Streptomyces vector capable of carrying large inserts. This

vector was chosen in preference to the shuttle cosmid, used in clone bank preparation, to avoid deletion of the inserted sequences when inserting clone 8a directly into Streptomyces.

Fragment 8a was digested with BglII and a 13 kb BglII subfragment which contained the actinorhodin Gene III hybridizing sequence and a 10 kb BglII subfragment containing the actinorhodin Gene I hybridizing sequence were isolated. These subfragments were designated fragment 2 and fragment 7, respectively, as shown in figure 7. Digestion of the 8a fragment with BamHI allowed isolation of a 1.6 kb BamHI subfragment containing the Gene III hybridizing sequences, this subfragment was designated fragment 4 (Figure 7). Fragments 2, 7 and 4 were cloned into vector p IJ943, which is another low copy number Streptomyces vector (available from David Hopwood, John Innes Institute). Insertion of DNA fragments into the Bgl II cloning site of pIJ 943 results in the loss of melanin production by the host bacterium, thus providing a convenient screen for insertion.

Two mutants of S. coelicolor which are blocked in actinorhodin Gene III (TK18) or Gene I (TK 17) were obtained from David Hopwood, John Innes Institute. Each of these mutants was transformed with pIJ 922, containing fragment 12 (27 kb), or p IJ 943, containing fragment 2, fragment 7 or fragment 4. All manipulations of Streptomyces, including transformations, were performed essentially as described in Hopwood et al. (1985) Genetic Manipulation of Streptomyces - A Laboratory Manual, The John Innes Foundation. The results of these complementation experiments are shown in Table 1. Those fragments containing sequences that hybridize to actinorhodin Gene III were found to complement the S. coelicolor TK18 Gene III mutant. Fragment 10 which did not contain sequences that hybridize to Gene III did not

complement the Gene III mutant. Similarly, fragment 7 which did not contain sequences that hybridized to the Gene I probe did not complement the Gene I mutant. Fragment 10 which contains sequences that hybridize to the Gene I probe appears to have complemented the TK17 Gene I mutant, however the interpretation of the results of Table 1 is complicated since it has been found that the S. coelicolor TK17 Gene I mutant displays a "leaky" phenotype. Actinorhodin produciton was assayed using methods described in Malpartida and Hopwood, 1984, and Thompson et al. (1980) Nature 286:525-527.

Example 4: Introduction of Streptomyces sp. B41-146 DNA fragments into S. lividans

S. lividans contains the genes for production of actinorhodin, but does not normally produce this antibiotic. A strain of this species, S. lividans TK24 (Keiser et al. (1982) Mol. Gen. Genet. 185:223-228), was transformed with pIJ943 containing fragment 7 inserted in either orientation as well as with pIJ922 containing fragment 12 inserted in either orientation.

All S. lividans TK24 transformed with pIJ943 containing fragment 7 unexpectedly produced antinorhodin. Production of the antibiotic in transformants was found to be independent of the orientation of fragment 7. Fragment 7 therefor contains a DNA region that effects "switch-on" of silent actinorhodin genes in S. lividans. This result also strongly suggests that fragment 7 contains a DNA region that is involved in regulation of the production of milbemycin in Streptomyces sp. B41-146.

It was also found that introduction of fragment 12 into S. lividans, although only in one orientation effected production of actinorhodin.

A S. coelicolor mutant defective in Gene II actinorhodin, the putative regulatory gene for actinorhodin synthesis (Rudd and Hopwood (1979) J. Gen. Microbiol. 114:35-43; and Malpartida and Hopwood, 1986), was transformed with pIJ943 containing fragment 7. The resultant S. coelicolor transformants produced actinorhodin, indicating that milbemycin sequences in fragment 7 can also function in place of actinorhodin Gene II. by analogy to the actinorhodin genes, the milbemycin activating gene in fragment 7 is termed milbemycin Gene II.

It is interesting to note that even though the fragment 4 sequences functionally complement actinorhodin Gene II, no hybridization between fragment 7, milbemycin Gene II and actinorhodin Gene II sequences were detected. Sequences in fragment 7 are thus not structurally similar to those in Gene II.

Example 5: Extending the Streptomyces sp. B41-146 milbemycin gene cloned region to the right of fragment 8a

Since it appeared that fragment 7, which is a the far right end of 8a (Figure 6), contained a potential regulatory region for milbemycin production, it was possible that the milbemycin gene cluster extended to the right of sequences in fragment 8a. In order to clone Streptomyces sp. B41-146 regions to the right of fragment 8a, a 1.9 kb BamHI subfragment of 8a (fragment 14, Figure 6) was selected for use as a hybridization probe to a Streptomyces sp. B41-146 clone library. A second cosmid library was prepared with 20-30 kb DNA fragments from a partial Sau3a digest of B41-146 DNA and which were inserted into the cosmid vector pIMS6026. This second clone library was screened for hybridization to labelled fragment 14 probe cloned into the vector PGB2,

obtained from Y. Nagamine. This vector was used because it showed no sequence homology to several commonly used E. coli plasmids, including pIMS6026 (Churchward, et al. (1984) Gene 31:165-171). In this case, Southern hybridization was carried out at 50°C with prehybridization for 3 hrs with 6xSSC/50% formamide/0.1% SDS/50µg/ml herring sperm DNA, and hybridization overnight using the same solution. Four sequential 1 hr washes were used. The first with 6xSSC/50% formamide; the second with 2xSSC/50% formamide; the third with 1xSSC/50% formamide; and the fourth with 0.5xSSC/50% formamide.

Four clones (out of 1,000 screened) were found to hybridize to the fragment 14 probe. Restriction enzyme analysis of these four clones, indicated that only two, pPOD1062 and pPOD1064, extended in sequence to the right of 8a (figure 8), The inserts of these clones were designated fragments 62 and 64, respectively, and positioning of these fragments with respect to fragment 8a and each other is shown in Figure 8.

Fragments 10, 8b, 8a, 62 and 64 define a Streptomyces sp. B41-146 region of about 62kb in length. It is believed that this DNA region contains the complete gene cluster for milbemycin production. A composite fragment MC (approximately 62 kb) which contains the contiguous DNA of the milbemycin gene cluster is constructed from fragments 10, 8b, 8a, 62 and 64 by appropriate restriction enzyme cleavage and ligation steps with intermediate fragments and the 62 kb MC fragment isolated by conventional DNA gel separation techniques.

Example 6: Insertional inactivation of Streptomyces sp. B41-146 milbemycin biosynthesis genes with cloned fragments

Confirmation that the cloned region (approximately 62 kb) contained genes for milbemycin production was obtained by insertional inactivation of the milbemycin genes of Streptomyces sp. B41-146 using fragments of the cloned region in homologous recombination experiments.

These experiments were based on the techniques employed by Chater and Burton (1983) Gene 26:67-78. Basically, subfragments of the "milbemycin cluster" cloned region were inserted into an attachment site deleted defective phage vector. The recombinant phage vectors were then used to infect milbemycin producing Streptomyces sp. B41-146. Phage insertion into the chromosome was effected by homologous recombination events mediated by the homology of the inserted subfragment to the B41-146 chromosome. It is expected that only subfragments which are internal to a transcription unit will disrupt expression and result in a (milbemycin nonproducing) mutant phenotype (Chater and Burton, 1983). The resultant lysogens were cultured and subcultured several times on thiostrepton containing medium, and the surviving thiostrepton resistant bacteria were assayed for milbemycin production.

The phage vector used was phage pPOD9, the restriction map of which is shown if Figure 9. This vector is derived by insertion of the tsr (thiostrepton resistance) gene (Thompson et al. (1982) Gene 20:51-62) on a BclI-BamHI fragment into the BamHI site in the Tc gene of the ØC31 derivcative ØC31πW17, in which the attachment site is deleted (Chater et al. (1981) Gene 15:249-256). pPOD9 contains the tsr gene adjacent to a unique BamHI site.

Fragment 4 (1.6 kb BamHI fragment) which contains milbemycin Gene III was cloned into pPOD9 in both orientations to give phages pPOD10 and pPOD11 (Figure 10). Fragment 16 (1.7 kb BamHI fragment) adjacent to Gene I was cloned to produce phage pPOD12 (Figure 11). Fragment 14 (1.9 kb BamHI fragment) was also cloned into pPOD9 in both orientations to give phages pPOD1071 and pPOD1072 (Figures 12 and 13). These phages were used to infect wild-type Streptomyces sp. B41-146, and in each case individual thiostrepton resistant lysogens were assayed for milbemycin production.

Contrary to expectations it was found some lysogens resulting from infection with pPOD10 and pPOD11, which contain the entire milbemycin Gene III transcription unit, no longer produced milbemycin. Milbemycin produciton phenotype of individual lysogen was initially tested on plates assaying nematode killing (vide infra). Phenotypes were confirmed by abnalysis of culture broth extracts for milbemycins (vide infra). In view of these unexpected results, DNA was prepared form several individual lysogens and examined by Southern blotting with several probes, in order to determine the structure of the DNA that had been inserted. It was found, as exemplified for lysogen pPOD11/6 (which resulted from "insertion" of pPOD11), that althought the tsr gene had been incorporated from the phage, the milbemycin gene cluster had been disrupted by deletion of not only part of the phage DNA but also the milbemycin Gene III duplicate copy as well as chromosomal DNA further to the right of the site of recombination including sequence with the milbemycin Gene I region. The deletion which occurred in pPOD11/6 (non-producing lysogen) compared to the expected insertion are shown in Figure 10.

Similarly, infection of Streptomyces sp. B41-146 with pPOD12 resulted in lysogens defective for milbemycin production. Again, deletions of phage and

chromosomal DNA were observed on DNA analysis of selected lysogens. For example, Figure 11 compares the deletion in pPOD12/13 to the expected phage insertion.

After infection of Streptomyces sp. B41-146 with pPOD1071, only about 10% of the resultant lysogens were defective for milbemycin production, while infection with pPOD1072 resulted in a lysogen population with 68% loss of milbemycin production. Figure 12 compares the expected insertion of pPOD1071 to the structure of lysogen pPOD1071/4(15). This lysogen, which suffered only a deletion of Ø31 sequences, retains production of milbemycin. Figure 13 compares the expected insertion of pPOD1072 with the structure of lysogen pPOD1072/2(10 which does not produce milbemycin. Again, a deletion of phage and chromosomal DNA was found in this lysogen.

Thus, the infection experiments with phage DNA containing fragments from the milbemycin gene clones has shown that these fragments do contain genes essential for milbemycin production, since deletions in the chromosomal region at or near the location of these genes result in loss of milbemycin production.

The phage infection technique can therefore be used to creat a variety of milbemycin non-producing mutants by DNA deletions throughout the milbemycin biosynthetic cluster region. These mutants can be used in order to further investigate the genetics of milbemycin production and delineate other specific milbemycin genes within the milbemycin cluster region.

Example 7: Assays for milbemycin production

A plate assay for milbemycin production by bacteria was developed based on the nematocidal activity of milbemycin, and is herein exemplified for milbemycin production by Streptomyces sp. B41-146.

Streptomyces sp. B41-146 are plated on C agar medium (Goegelman et al. (1982) EPO Patent 058 518) at a density which allows formation of separate single colonies. A suspension of nematodes (Caenorhabditis elegans) is added to the test plate containing bacterial colonies. Plates are initially examined for nematode density and viability and again after 3-10 hours for nematode killing.

C. elegans is cultured as described in Brenner (1974) Genetics 77:71-94. NG agar is seeded with the E. coli uracil alexotroph OP50 in order to produce a bacterial lawn after incubation at 37°C for 18 hours.

A single hermaphrodite nematode (C. elegans strain N2) is added tot he bacterial lawn and the plate is incubated for 5 days at room temperature. After incubation the plate contains a dense population of C. elegans, which is then harvested using distilled water.

Milbemycin production was also assayed using an HPLC method described in Takiguchi et al. (1983) J. Antibiotics 36:502-508.

Seed medium (50 ml) in a 250 ml baffled flask was inoculated with 50µl of spores of the strain to be examined. The culture was incubated with shaking at 28°C for 12 days (Takiguchi et al., 1983).

After incubation the total culture was extracted with acetone. Acetone extracts were concentrated 10-fold and 100µl aliquots of the concentrated

extract were applied to a C18 reverse-phase HPLC column for analysis. HPLC conditions used are those described in Takiguchi et al., 1983. Column effluent was monitored at 240 nm.

Those skilled in the art will appreciate that the invention described herein and the methods described are susceptible to variations and modification other than as specifically described. It is to be understood that the invention includes all such variations and modifications which fall within its spirit and scope.

Table I: Complementation of Act- mutants with fragments cloned from Streptomyces sp. B41-146

	Streptomyces sp. B41-146 Fragments			
Act mutant	12 (27kb)	2 (13 kb)	7 (10kb)	4 (1.6 kb)
TK 17	not tested	-	(+)	not tested
TK 18	+	+	-	+

+ = complementation - = no complementation

(+) = complementation variable; TK17 mutation is leaky

We claim:

1. A method for isolating a gene involved in the biosynthesis of a first polyketide antibiotic which comprises the steps of:

   a. preparing a clone library wherein each clone contains a fragment of DNA from a microorganism which produces said first polyketide antibiotic;

   b. screening said clone library for hybridization to a nucleic acid probe molecule which comprises the nucleic acid sequence of at least a part of a gene involved in the biosynthesis of a second polyketide antibiotic; and

   c. selecting those clones which hybridize to said nucleic acid probe molecule thereby isolating a clone which contains a fragment of DNA which comprises said gene involved in the biosynthesis of said first polyketide antibiotic.

2. A method according to claim 1 wherein said microorganism is a bacterium of the genus Streptomyces.

3. A method according to claim 1 wherein said nucleic acid probe comprises the nucleic acid sequence of at least a part of a gene involved in the biosynthesis of actinorhodin.

4. A method according to claim 3 wherein said nucleic acid probe comprises the nucleic acid sequence of at least a part of the actinorhodin Gene III.

5. A method according to claim 3 wherein said nucleic acid probe comprises the nucleic acid sequence of at least a part of the actinorhodin Gene I.

6. A method according to claim 1 wherein said nucleic acid probe comprises the nucleic acid sequence of at least a part of a gene involved in the biosynthesis of milbemycin.

7. A method according to claim 6 wherein said nucleic acid probe comprises the nucleic acid sequence of at least a part of the milbemycin Gene III.

8. A method according to claim 6 wherein said nucleic acid probe comprises the nucleic acid sequence of at least a part of the milbemycin Gene I.

9. A method for producing a first polyketide antibiotic in a naturally non-producing strain of a bacterium of the genus Streptomyces which comprises the steps of:

a. isolating a DNA fragment which comprises the clustered biosynthetic genes for said first polyketide antibiotic from a strain of a bacterium of the genus Streptomyces which produces said first polyketide antibiotic wherein said isolation comprises the steps of:

i. preparing a clone library wherein each clone contains a fragment of DNA from a microorganism which produces said first polyketide antibiotic;

ii. screening said clone library for hybridization to a nucleic acid probe molecule which comprises the nucleic acid sequence of at least a part of a gene involved in the biosynthesis of a second polyketide antibiotic;

iii. selecting those clones which hybridize to said nucleic acid probe molecule thereby isolating a clone which comprises a first DNA fragment which comprises at least part of the biosynthetic gene cluster of said first polyketide antibiotic;

iv. testing said first selected DNA fragment which comprises at least part of the biosynthetic gene cluster of said first polyketide antibiotic for the ability to direct synthesis of said first polyketide antibiotic in said non-producing strain of Streptomyces;

v. rescreening said clone library or a second clone library with a nucleic acid probe which comprises the right or left terminal region of said first selected DNA fragment;

vi. selecting those clones which hybridized to the right or left terminal region of said first selected DNA fragment thereby isolating a clone which comprises a second DNA fragment which

comprises chromosomal DNA sequences to the right or left of said first selected clone;

vii. repeating said testing, rescreening and selection steps employing said second selected DNA fragment and subsequent selected DNA fragments until said DNA fragment which comprises the clustered biosynthetic genes for said first polyketide antibiotic is isolated as ascertained by production of said first polyketide antibiotic in said nonproducing strain of Streptomyces in said testing step; and

b. introducing said cloned fragment containing said cluster of biosynthetic genes into said non-producing strain of a bacterium of the Streptomyces, thereby producing said polyketide antibiotic in said non-producing strain of Streptomyces.

10. A method according to claim 9 wherein said nucleic acid probe comprises the nucleic acid sequence of at least a part of a gene involved in an early step of the biosynthesis of said second polyketide antibiotic.

11. A method according to claim 9 wherein said nucleic acid probe comprises the nucleic acid sequence of at least a part of a gene involved in the biosynthesis of actinorhodin.

12. A method according to claim 11 wherein said nucleic acid probe comprises the nucleic acid sequence of at least a part of actinorhodin Gene III.

13. A method according to claim 11 wherein said nucleic acid probe comprises the nucleic acid sequence of at least a part of actinorhodin Gene I.

14. A method according to claim 9 wherein said nucleic acid probe comprises the nucleic acid sequence of at least a part of a gene involved in the biosynthesis of milbemycin.

15. A method according to claim 14 wherein said nucleic acid probe comprises the nucleic acid sequence of at least a part of milbemycin Gene III.

16. A method according to claim 14 wherein said nucleic acid probe comprises the nucleic acid sequence of at least a part of milbemycin Gene I.

17. A method according to claim 9 wherein said naturally non-producing strain of a bacterium of the genus Streptomyces is selected from the group of strains consisting of strains of Streptomyces lividans, strains of Streptomyces ambofaciens, strains of Streptomyces coelicolor and strains of Streptomyces avermitilis.

18. A method according to claim 9 wherein said first polyketide antibiotic is a milbemycin.

19. A method according to claim 18 wherein said naturally non-producing strain of a bacterium of the genus Streptomyces is a strain of Streptomyces lividans.

20. A method according to claim 9 wherein said first polyketide antibiotic is an avermectin.

21. A method according to claim 20 wherein said naturally non-producing strain of a bacterium of the genus Streptomyces is a strain of Streptomyces lividans.

22. A method for producing a milbemycin in a naturally non-milbemycin producing strain of a bacterium of the genus Streptomyces which comprises introducing into said non-producing strain a DNA fragment which comprises the DNA fragment MC.

23. A method according to claim 22 wherein said naturally non-milbemycin producing strain is a strain of Streptomyces lividans.

24. A method for activating expression of a polyketide antibiotic biosynthetic gene cluster in a bacterial strain of the genus Streptomyces which strain contains the biosynthetic genes sufficient for production of said polyketide antibiotic but lacks a functional polyketide antibiotic biosynthesis activator gene which comprises introducing into said bacterial strain a DNA fragment which comprises milbemycin Gene II.

25. A method according to claim 24 wherein said bacterial strain is a strain of Streptomyces lividans.

26. A method according to claim 25 wherein said polyketide antibiotic is actinorhodin.

27. A method according to claim 24 wherein said bacterial strain is a strain of Streptomyces coelicolor and said polyketide antibiotic is actinorhodin.

28. A method according to claim 24 wherein said bacterial strain is a strain of Streptomyces avermitilis and said polyketide antibiotic is an avermectin.

29. A vector which comprises a DNA fragment consisting essentially of the milbemycin biosynthetic gene cluster.

30. A bacterial strain containing the vector of claim 29.

31. A vector according to claim 30 wherein said DNA fragment is the fragment MC.

32. A bacterial strain containing the vector of claim 31.

33. A vector which comprises a DNA molecule consisting essentially of the DNA sequence encoding a milbemycin gene selected from the group consisting of milbemycin Gene I, milbemycin Gene II, and milbemycin Gene III.

34. A bacterial strain containing the vector of claim 33.

35. A vector according to claim 29 wherein said DNA molecule is a DNA fragment selected from the group of DNA fragments consisting of fragment

1, fragment 2, fragment 3, fragment 4, fragment 5, fragment 6, fragment 7, fragment 8a, fragment 8b, fragment 10, fragment 12, fragment 14, fragment 16, fragment 62, and fragment 64.

36. A bacterial strain containing the vector of claim 35.

37. A hybridization probe molecule consisting essentially of the milbemycin biosynthetic gene cluster.

38. A hybridization probe molecule that is fragment MC.

39. A hybridization probe consisting essentially of the DNA sequence encoding a milbemycin gene selected from the group of milbemycins genes consisting of milbemycin Gene I, milbemycin Gene II and milbemycin gene III.

40. A hybridization probe that is a DNA fragment selected from the group of DNA fragments consisting of fragment 1, fragment 2, fragment 3, fragment 4, fragment 5, fragment 6, fragment 7, fragment 8a, fragment 8b, fragment 10, fragment 12, fragment 14, fragment 16, fragment 62, and fragment 64.

41. A hybridization probe, the sequence of which is derived from the sequence of a fragment selected from the group of fragments consisting of fragment 1, fragment 2, fragment 3, fragment 4, fragment 5, fragment 6, fragment 7, fragment 8a, fragment 8b, fragment 10, fragment 12, fragment 14, fragment 16, fragment 62, and fragment 64.

42. A DNA fragment selected from the group of DNA fragments consisting of fragment 1, fragment 2, fragment 3, fragment 4, fragment 5, fragment 6, fragment 7, fragment 8a, fragment 8b, fragment 10, fragment 12, fragment 14, fragment 16, fragment 62, fragment 64 and fragment MC.

POLYKETIDE PATHWAY

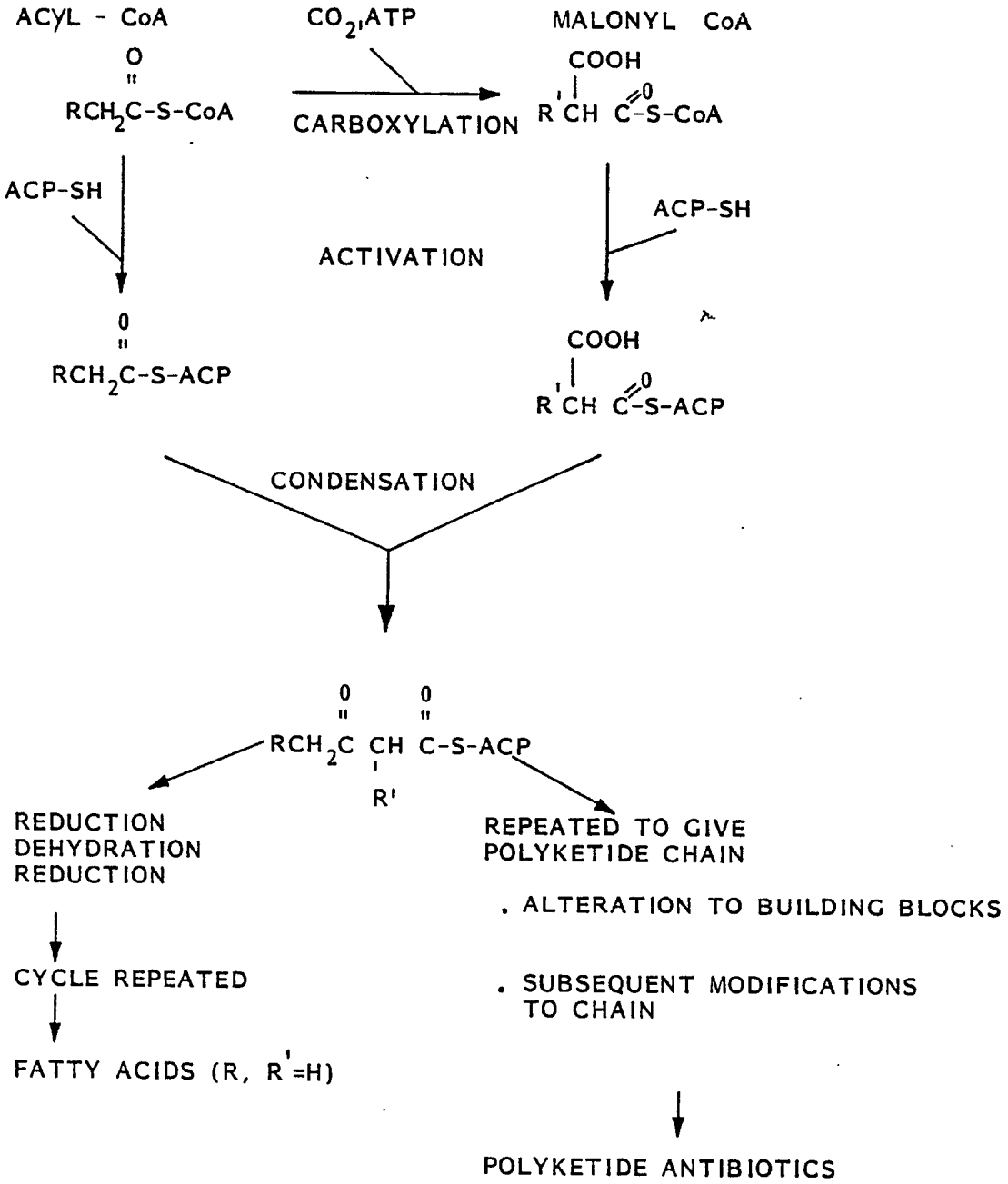
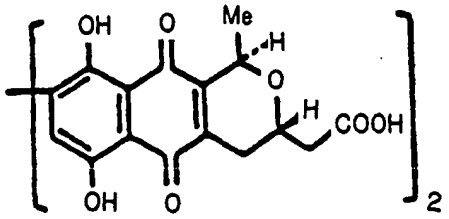
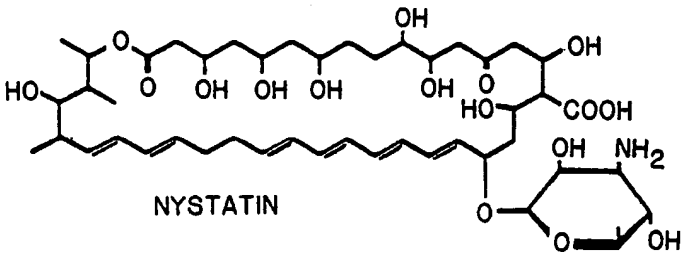


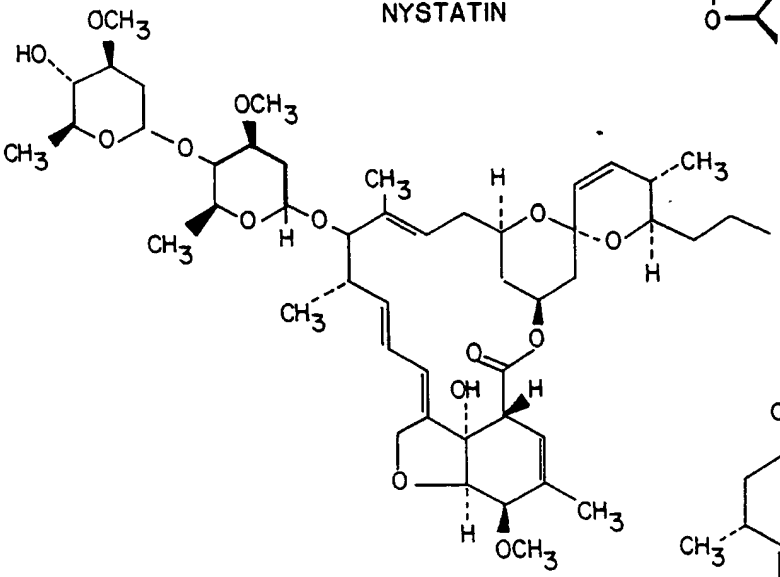
FIG. 1



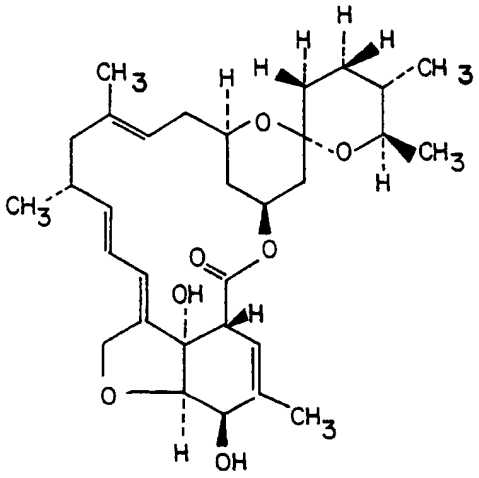
ACTINORHODIN



NYSTATIN



AVERMECTIN A_{1a}



MILBEMYCIN $\alpha_1(A_3)$

FIG. 2

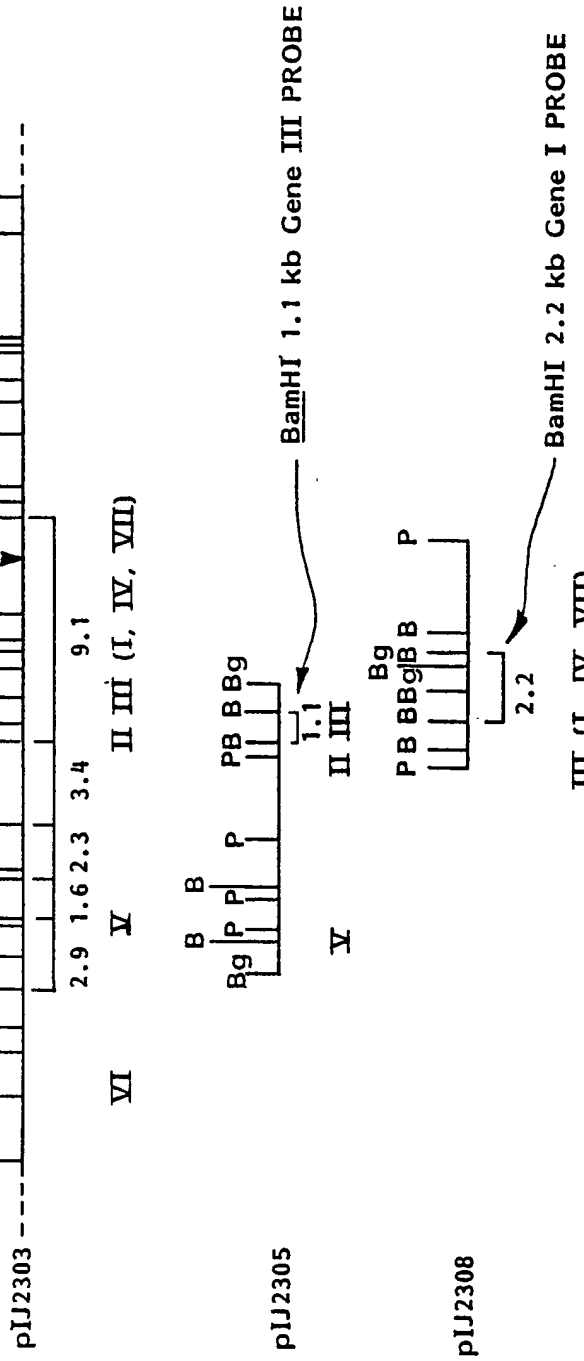
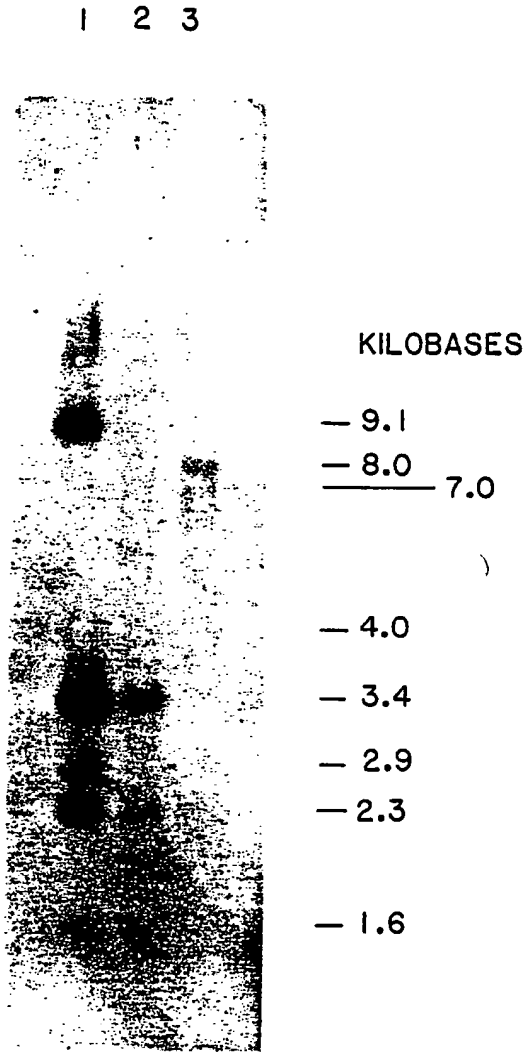
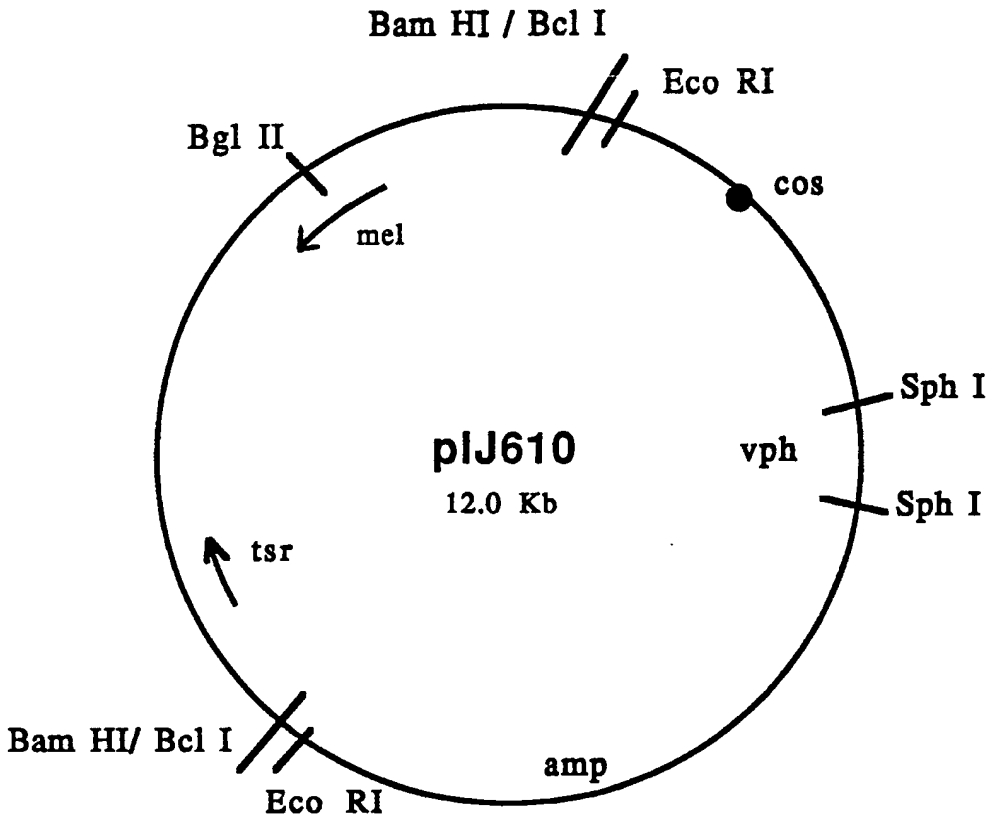

pIJ2303
pIJ2305
pIJ2308
BamHI 1.1 kb Gene III PROBE
BamHI 2.2 kb Gene I PROBE
B=BamHI
P=PstI
Bg=BglII
EV=EcoRV
EI=EcoRI

FIG. 3



1. S. coelicolor
2. S. lividans
3. Streptomyces sp. B41-146 (Milbenycin producer)

FIG. 4



vph = viomycin phosphotransferase
amp = ampicilin
tsr = thiostrepton
mel = melanin

FIG. 5

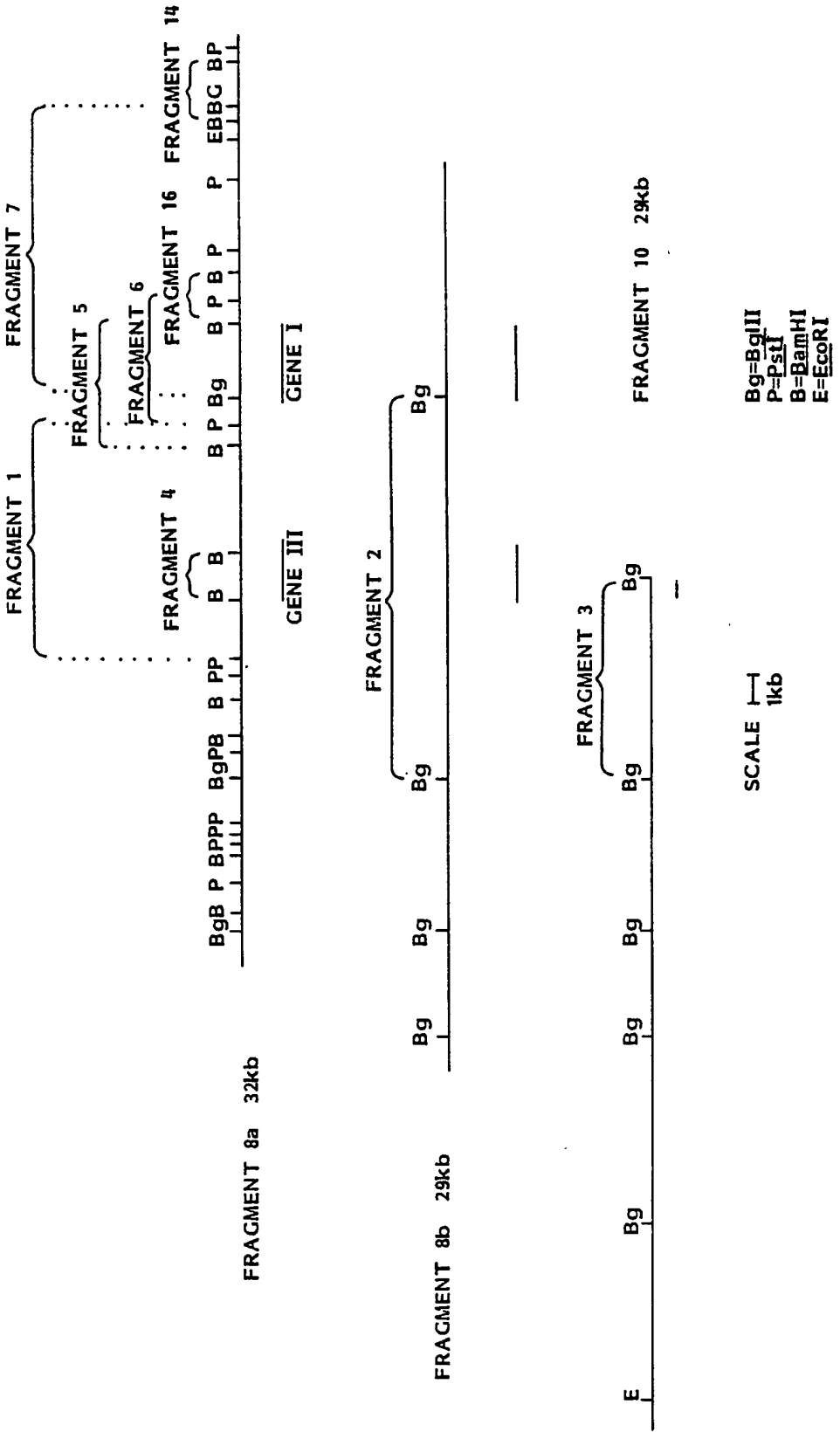

FRAGMENT 1
FRAGMENT 7
FRAGMENT 5
FRAGMENT 6
FRAGMENT 4
FRAGMENT 16
FRAGMENT 14
BgB P BPPP BgPB B PP B B B P Bg B P B P P EBBG BP
GENE III
GENE I
FRAGMENT 8a 32kb
FRAGMENT 2
Bg
FRAGMENT 8b 29kb
FRAGMENT 3
FRAGMENT 10 29kb
E
SCALE 1kb
Bg=BglII
P=PstI
B=BamHI
E=EcoRI

FIG. 6

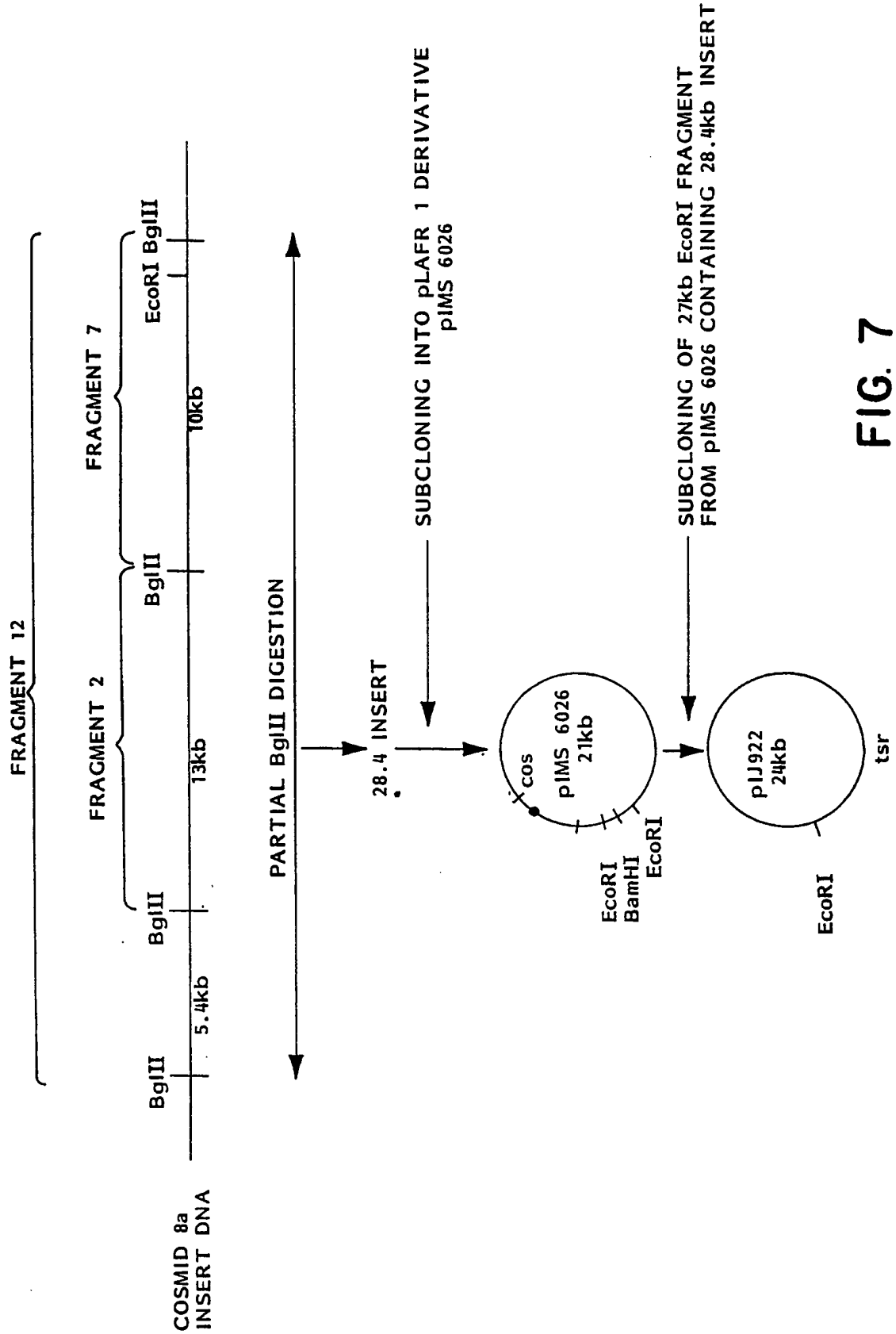

COSMID 8a
INSERT DNA
BglII
5.4kb
BglII
13kb
BglII
10kb
EcoRI BglII
FRAGMENT 12
FRAGMENT 2
FRAGMENT 7
PARTIAL BglII DIGESTION
28.4 INSERT
SUBCLONING INTO pLAFR 1 DERIVATIVE
pIMS 6026
cos
pIMS 6026
21kb
EcoRI
BamHI
EcoRI
SUBCLONING OF 27kb EcoRI FRAGMENT
FROM pIMS 6026 CONTAINING 28.4kb INSERT
pIJ922
24kb
tsr
EcoRI

FIG. 7

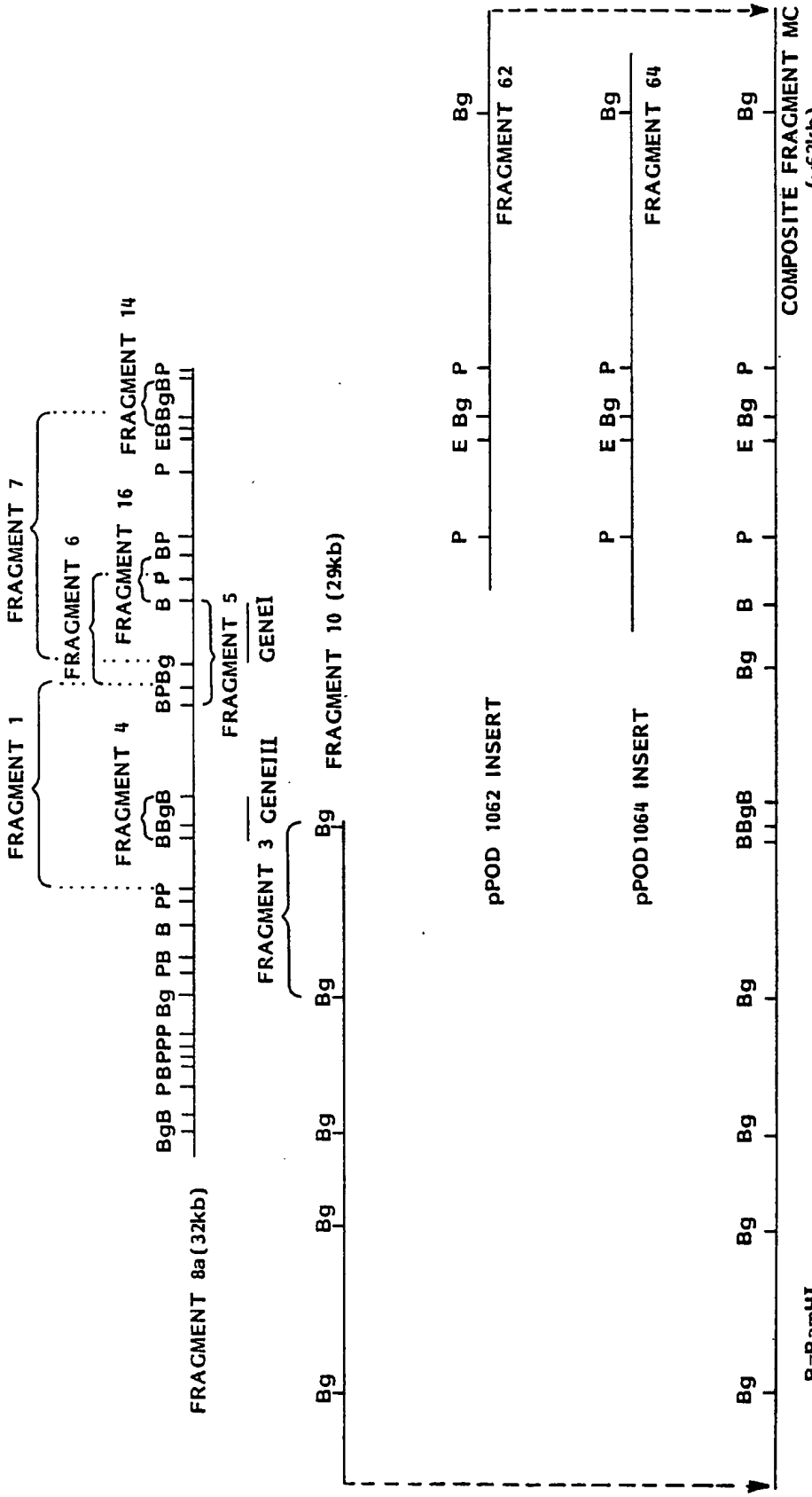

FRAGMENT 1
FRAGMENT 7
FRAGMENT 6
FRAGMENT 4
FRAGMENT 16
FRAGMENT 14
FRAGMENT 5
GENEI
FRAGMENT 3
GENEIII
FRAGMENT 10 (29kb)
FRAGMENT 8a(32kb)
pPOD 1062 INSERT
pPOD1064 INSERT
FRAGMENT 62
FRAGMENT 64
COMPOSITE FRAGMENT MC (~62kb)
B=BamHI
Bg=BglII
P=PstI
E=EcoRI

FIG. 8

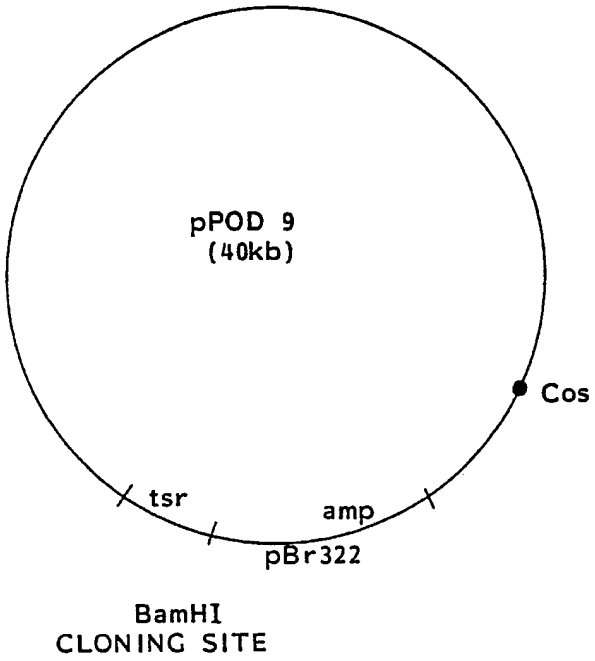


FIG. 9

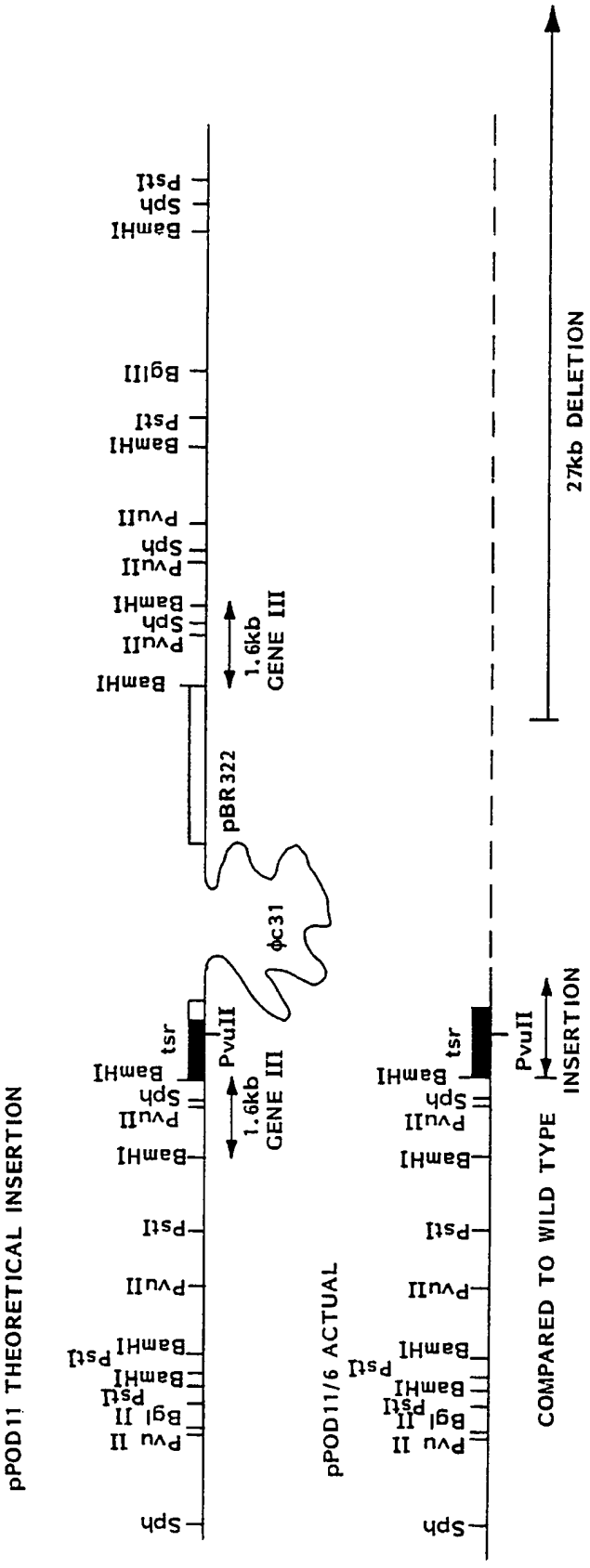

pPOD11 THEORETICAL INSERTION
pPOD11/6 ACTUAL
COMPARED TO WILD TYPE
27kb DELETION
INSERTION
pBR322
ϕc31
tsr
PvuII
1.6kb
GENE III
BamHI
SphI
PvuII
PstI
BglII
PvuII

FIG. 10

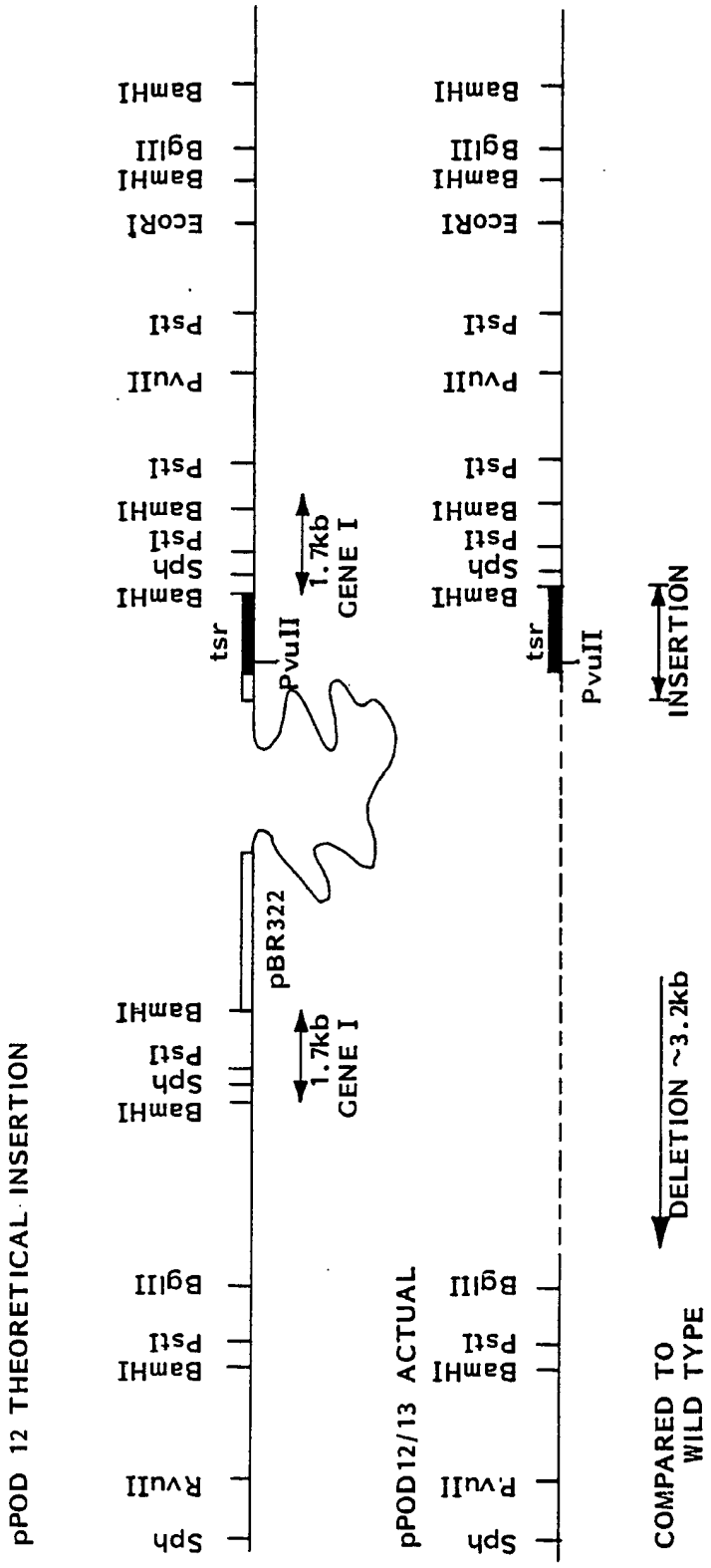

pPOD 12 THEORETICAL INSERTION
pPOD12/13 ACTUAL
COMPARED TO WILD TYPE
pBR322
tsr
PvuII
1.7kb
GENE I
INSERTION
DELETION ~3.2kb
BamHI
BglII
PstI
SphI
EcoRI
PvuII
SphI

FIG. II

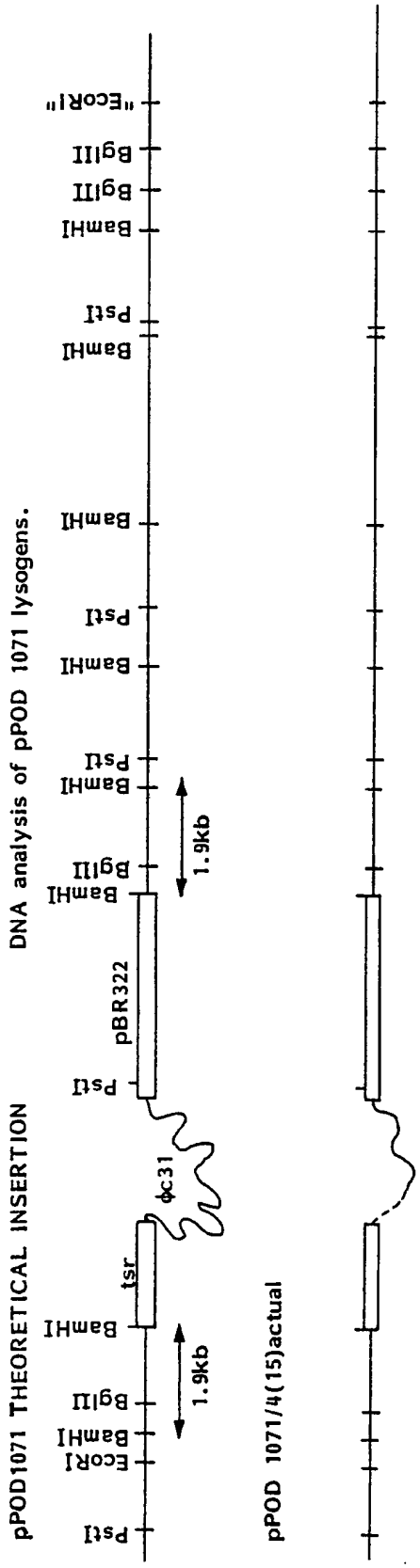

DNA analysis of pPOD 1071 lysogens.
pPOD1071 THEORETICAL INSERTION
pPOD 1071/4(15)actual
PstI
EcoRI
BamHI
BglII
"EcoRI"
BamHI
BglII
BamHI
PstI
BamHI
BamHI
PstI
BamHI
PstI
BamHI
BglII
BamHI
1.9kb
pBR322
PstI
ϕc31
tsr
BamHI
1.9kb
BglII
BamHI
EcoRI
PstI

FIG. 12

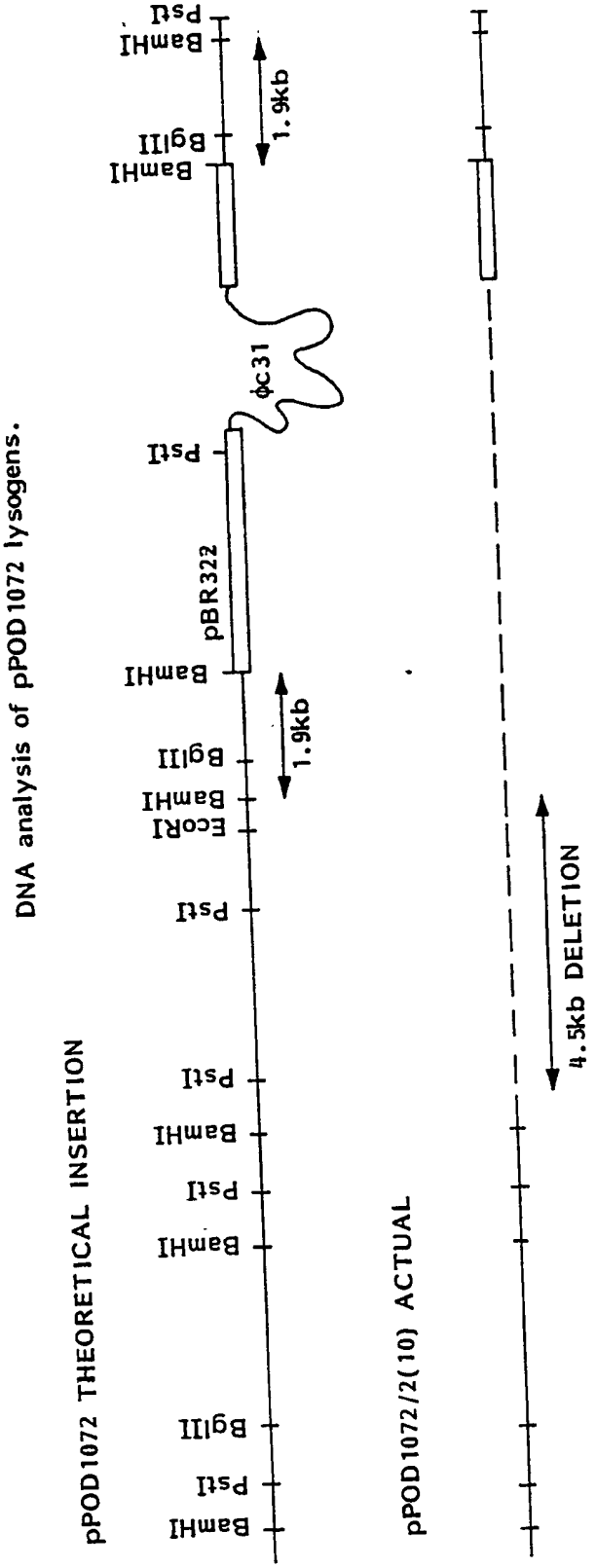

DNA analysis of pPOD1072 lysogens.
pPOD1072 THEORETICAL INSERTION
pPOD1072/2(10) ACTUAL
BamHI
PstI
BglII
PstI
BamHI
PstI
BamHI
PstI
EcoRI
BamHI
BglII
1.9kb
BamHI
pBR322
PstI
φc31
BamHI
BglII
1.9kb
4.5kb DELETION

FIG. 13

INTERNATIONAL SEARCH REPORT

International Application No PCT/US 86/02738

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) [6]

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC[4]: C 12 N 15/00; C 12 Q 1/68; C 12 N 1/20//(C 12 N 1/20; C 12 R 1: 465)

II. FIELDS SEARCHED

Minimum Documentation Searched [7]

Classification System	Classification Symbols
IPC[4]	C 12 N; C 12 Q

Documentation Searched other than Minimum Documentation to the Extent that such Documents are included in the Fields Searched [8]

III. DOCUMENTS CONSIDERED TO BE RELEVANT [9]

Category *	Citation of Document, [11] with indication, where appropriate, of the relevant passages [12]	Relevant to Claim No. [13]
Y	Gene, volume 26, 1983, Elsevier Science Publishers, (Amsterdam, NL), K.F. Chater et al.: "Mutational cloning in Sreptomyces and the isolation of antibiotic production genes", pages 67-78 see summary, introduction; page 72, line 8 - page 74, line 12; page 76 cited in the application	24,25
Y	EP, A, 0118367 (MERCK & CO. INC.) 12 September 1984 see abstract; page 10, line 28 - page 11, line 14; example 2	24
A		20,28
A	Nature, volume 309, no. 5967, 31 May 1984, (London, GB), F. Malpartida et al.: "Molecular cloning of the whole biosynthetic pathway of a Streptomyces antibiotic and its expression in a heterologous host", pages 462-464	

./.

* Special categories of cited documents: [10]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
9th April 1987	19 MAY 1987
International Searching Authority	**Signature of Authorized Officer**
EUROPEAN PATENT OFFICE	M. VAN MOL

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No
	see the whole document, particularly the last two paragraphs cited in the application	24,25
	--	
P,Y	Biotechnology, volume 4, no. 3, March 1986, R. Stanzak et al.: "Cloning and expression in Streptomyces lividans of clustered erythromycin biosynthesis genes from Streptomyces erythreus", pages 229-232 see the whole document cited in the application	24,25
	--	
P,A	Biotechnology, volume 4, no. 9, September 1986, J.T. Fayerman: "New developments in gene cloning in antibiotic producing microorganisms", pages 786-789 see the whole document, particularly page 788, column 1, lines 2-17	1-5

ANNEX TO THE INTERNATIONAL SEARCH REPORT ON

INTERNATIONAL APPLICATION NO. PCT/US 86/02738 (SA 15709)

This Annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report. The members are as contained in the European Patent Office EDP file on 21/04/87

The European Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
EP-A- 0118367	12/09/84	JP-A- 59169496	25/09/84